STUDIES ON INTERMEDIARY IRON METABOLISM

4. HAEMOGLOBIN VALUE, SERUM IRON AND IRON BINDING CAPACITY IN NORMAL AND CASTRATED RATS

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(From the N.S.W. Red Cross Society's Blood Transfusion Service).

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A sex difference in erythrocyte count and haemoglobin concentration develops in man at the time of puberty and is maintained throughout life (Rud, 1922; Walsh, Arnold, Lancaster, Coote and Cotter, 1953). A similar difference between the serum iron values of adult men and women was reported in an earlier paper of this series (Kaldor, 1953b). A study of normal and hysterectomised women (Vahlquist, 1950) showed that these differences are independent of catamenic blood loss.

A sex difference in erythrocyte count and in haemoglobin concentration has been found in many other species (Gordon and Charipper, 1947). Steinglass, Gordon and Charipper (1941) found higher erythrocyte and haemoglobin values in male rats than in females. Castration produces an equalization of this sex difference. Creskoff, Fitz-Hugh and Farris (1942), on the other hand, reported higher values in females than in males.

Although rats have been used extensively in recent investigations on body iron metabolism, no systematic data on serum iron and iron binding capacity values are available for this animal.

This paper reports measurements of haemoglobin, serum iron and iron binding capacity in normal and castrated rats of both sexes.

Materials and Methods.

Some two hundred male and female rats, between 6 and 10 weeks old and 60 to 120 gm. body weight, were obtained from the same breeding stock. Under light anaesthesia castration was performed on approximately 50 p.c. of the male and on the same proportion of the female animals. All were fed a diet of commercial rat food and were given unrestricted supplies of drinking water.

The animals were sacrificed when blood was required for tests. After 12 hours' fasting they were anaesthetized with ether and the chest wall opened parasternally. Blood was withdrawn from the pulsating heart with a syringe and needle. The volume obtained ranged between 2·5 and 3·0 ml. for 100 gm. body weight, or 40 and 50 p.c. of the calculated total blood volume (Scott and Barcroft, 1924).

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Haemoglobin was measured colorimetrically by the technique of Walsh *et al.* (1953).

Serum iron and iron binding capacity were measured as described in a previous paper (Kaldor, 1953a). The colorimeter used for this purpose was fitted with a cuvette of smaller capacity than the original. This modification and the relatively high serum iron values in rats made it possible to reduce the amount of serum required for a single assay to 1 ml. With a few exceptions, measurements were made on duplicate serum aliquots. When insufficient blood was obtained from an animal to allow the test to be performed in duplicate, the sera of two or three rats were pooled in equal proportions. The same procedure was followed in the measurement of iron binding capacity.

RESULTS.

The four groups—normal males, normal females, castrated males and castrated females—were tested in three batches, sacrificed at two, five and seven months respectively from the time of castration. The results are recorded in Table 1.

TABLE 1.

Haemoglobin, serum, iron and iron binding capacity values.

Group	Batch number	Number of animals	Mean weight gm.	Haemoglobin concentration gm. per 100 ml. blood Mean*	St. dev.	Serum iron micrograms per 100 ml. serum Mean*	St. dev.	Total iron binding capacity 100 ml. serum Mean*	St. dev.
Normal Males	1	10	200	14·87±0·34	1·09	245±22	59	483±34	68
	2	11	290	14·22±0·22	0·75	178±11	36	—	—
	3	18	310	15·32±0·21	0·87	160± 8	34	449±17	57
Normal Females	1	14	160	14·32±0·23	0·85	335±14	40	456±28	49
	2	11	210	14·66±0·23	0·74	291±25	81	—	—
	3	16	210	14·68±0·12	0·59	258±14	52	374±16	37
Castrated Males	1	4	230	13·64±0·24	0·49	213±35	61	—	—
	2	11	290	14·89±0·15	0·52	201±16	53	—	—
	3	35	280	14·20±0·14	0·80	168± 4	21	405±10	38
Castrated Females	1	10	220	14·04±0·28	0·88	239±30	67	—	—
	2	11	240	15·31±0·21	0·68	202±13	41	—	—
	3	20	240	14·46±0·14	0·61	223±10	49	379± 9	26

* The figures shown are the mean, ± its standard error.

All groups gained weight until the fifth month, but there was little subsequent change.

There is no evidence of a consistent sex difference in the haemoglobin values or of consistent changes in these values with age. Castration produced no significant changes in the values in either sex.

In the serum iron values it will be seen that:

(1) In all three batches the mean values of the normal females are significantly higher than those of the normal males.

(2) The values decline with age in both normal male and normal female groups.

(3) Castration produced no significant changes in males. In females the mean values at the three intervals after castration are all less than those of normal females of corresponding age.

(4) The values do not significantly decline with age in either sex after castration.

Despite the low serum iron values in males, the total iron binding capacity values of both normal and castrated male groups are slightly higher than those of the corresponding female groups. There is some evidence of a decline of the values with age in normal males and females.

DISCUSSION.

The clear cut sex difference in the haemoglobin and serum iron values in man is unexplained. The possible influence of the gonads in establishing this difference was explored in these experiments on the rat because Steinglass *et al.* (1941) had reported the sex difference in this animal and its disappearance upon castration. However, no significant difference was found in the present work in the haemoglobin values of normal rats, nor did castration bring about any significant change in these values.

Since few measurements have previously been made to determine the physiological variations of serum iron and iron binding capacity values in the rat, these values are reported in some detail. In contradistinction to the haemoglobin values there was a well-marked sex difference in serum iron. The serum iron values of normal female rats were some 50 p.c. higher than those of normal males. No explanation for this difference can be offered at present, although Widdowson and McCance (1948) found more than twice as much iron stored in the livers of female rats than of males. The amount of storage iron is certainly one of the factors which determines the level of serum iron, and it may be pointed out that women, whose liver iron concentration is lower than that of men (Bruckman and Zondek, 1939) also have lower serum iron and haemoglobin values.

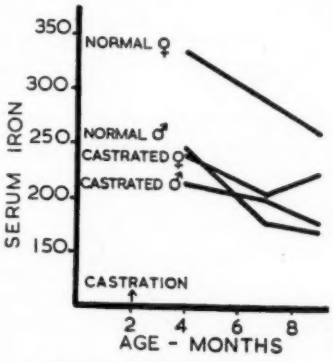

Fig. 1. Serum iron values.

A feature common to both normal male and female rats was the gradual decline of the serum iron value in the second and third batches as compared with the first. In five months the mean values decreased a total of 35 p.c. and 29 p.c. in males and females respectively.

As there was a definite sex difference in the serum iron value of normal rats, it might have been expected that castration would eliminate the difference. This actually occurred as is shown in Fig. 1. This was effected by a decrease of the serum iron values in females, the value of males being mostly unchanged.

The total iron binding capacity value is an estimate of the concentration of the specific iron binding protein. In man this protein is kept under normal conditions at a relatively constant level while the concentration of serum iron may vary considerably according to physiological requirements. Similarly, in all groups of rats tested, the mean total iron binding capacity values showed considerably less variation than did the serum iron values.

SUMMARY.

No sex difference was found in the haemoglobin values of normal rats.

Castration produced no significant changes in the haemoglobin values of either male or female rats.

The serum iron values of normal female rats were 50 p.c. higher than those of normal males.

Castration reduced the serum iron values of female rats but caused little change in males.

The serum iron values declined with increasing age in normal animals of both sexes, but not in castrated animals.

No significant variations of total iron binding capacity were observed in either male or female rats.

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RECOMBINATION BETWEEN NEUROTROPIC AND NON-NEUROTROPIC INFLUENZA VIRUS STRAINS

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In several previous papers recombination between a neurotropic influenza A virus strain NWS and several non-neurotropic influenza A viruses has been described (Burnet and Lind, 1951a,b; Burnet and Edney, 1951; Fraser and Burnet, 1952). Most of the work was done with the combination NWS/MEL, from which neurotropic strains of MEL serological character (N-M strains) were obtained as recombinants. These strains had a number of other differences from MEL as well as their encephalitogenic power, notably the capacity to produce haemorrhagic lesions in chick embryos and to be converted to the "indicator state" by simple heating. When interest was transferred to the MEL/WSE system, in which neither virus is neurotropic (Burnet and Lind, 1952), it became clear that the M+ recombinant obtained from this system was very similar to N-M. In fact the "non-neurotropic N-M strains," described by Burnet and Edney (1951), were indistinguishable from M+ strains. If we adopt the formulation (*vide infra*) that has been presented for the MEL/WSE interaction and represent the types concerned as

ABDF–CE (MEL), abdf–ce (WSE), ABDF–ce (M+), abdf–CE (WS—)

then the neuro-MEL (N-M) strains from the earlier work could be formulated as ABDF–ceg (g representing encephalitogenic power in mice and G the absence of that quality). Similarly, the NWS strain will be represented ab'(d)f–(e)eg. The extreme heat lability of this strain indicated by b' makes it impossible to demonstrate the existence of the phenotypic characters d and e, so they are indicated in brackets. The NWS/MEL reaction would then be represented

ABDF–CEG/ab'(d)f–(e) eg→ABDF–ceg.

On the other hand the neuro WSM, similarly obtained by Burnet and Lind (1951a) and characterized essentially by the combination of very high thermal resistance of its haemagglutinin (represented B') with neuropathogenicity, was not lethal to the chick embryo so that its origin must be represented

aB'dfcE–G/ab'(d)f(e)e–g→aB'dfcE–g
WSM NWS NWSM

[1]This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

It appears that in the first instance the recombination involved the transfer of the linkage groups CEG/(c)eg, but in the second only an interchange of G and g occurred.

It was thought that it would be of considerable interest to examine further examples of the transfer of neurotropism from one virus type to another in the light of experience gained with the system MEL/WSE in de-embryonated egg experiments. Special attention has been directed in the experiments to be reported, to the distribution of neuro-pathogenicity amongst the derivative strains.

Materials and Methods.

Virus strains.

The following virus strains were used:

WSE, WSM, NWS. Three substrains of the classical WS influenza type A strain. These strains have been through many egg and mouse passages and a full description of the characters of the lines maintained in this laboratory has been given by Burnet (1951a).

WS–. A strain obtained by recombination between type A strains Melbourne and WSE (Burnet, Fraser and Lind, 1953; Burnet and Lind, 1952). Fourth limit dilution passage material was used as stock.

Neuro-WSM (N-WSM). A strain obtained by recombination between NWS and WSM (Burnet and Lind, 1951a). Fourth limit dilution passage material was used as stock.

Neuro-Melbourne (N-M). A strain obtained by recombination between NWS and Melbourne (Burnet and Lind, 1951b). Fifth limit dilution passage material was used as stock.

Freshly harvested virus from a further allantoic passage of these stock viruses, at a dilution of 10^{-4}, was used as starting material for experimental work.

Diluents.

Saline. 9 gm. of NaCl made up to 1,000 ml. with distilled water.

Horse serum saline (HSS). Saline containing 10 p.c. normal horse serum.

Determination of marker characters.

There are seven *in vitro* and *in vivo* marker tests by means of which the strains used could be distinguished from one another. These tests are as follows:

Aa. Serological type. This was determined by means of haemagglutinin inhibition tests using appropriate hyperimmune rabbit sera. The method was based on that described by Burnet, Beveridge, Bull and Clark (1942). All viruses used were either of the serological character of Melbourne, designated by A, or of WS, designated by a.

BB'b'b. Heat resistance of haemagglutinin (H.A.). This was determined by heating small aliquots of virus fluid at certain temperatures for 30 minutes. After heating, the tubes were chilled quickly and the virus titrated for residual H.A. There is a considerable variation in heat resistance among the strains used, and this variation and the symbols given to the various levels of heat resistance are summarized in Table 1.

Cc. Conversion to indicator. When certain viruses are heated at 55° C. for 30 minutes they are converted to the indicator state, i.e., their haemagglutinin becomes susceptible to inhibition by high dilutions of a number of soluble mucoids. (See Burnet, 1951b.) Viruses which were converted to indicator under these conditions were given the symbol c, those which were not the symbol C. In these experiments semi-purified meconium (Curtain, French and

Pye, 1953) was used as haemagglutinin inhibitor and the titration method of Burnet (1948) was followed.

Dd. Inhibition of haemagglutinin by sheep mucoid. Viruses whose haemagglutinin, after simple heating at 55° C. for 30 minutes, was inhibited by sheep salivary mucoid (McCrea, 1951) were designated d. Those whose haemagglutinin was not thus inhibited were designated D.

TABLE 1.

Heat stability of haemagglutinin (H.A.).

Virus	Heated 30 min. at temp. 50° C.	55° C.	60° C.	65° C.	Symbol
NWS	+	−	−	−	b'
WSE	+	+	−	−	b
WS−	+	+	−	−	b
N-M	+	+	+	−	B
WSM	+	+	+	+	B'
N-WSM	+	+	+	+	B'

+=H.A. present. −=H.A. destroyed.

Ee. Invasiveness for the chick embryo. Certain of the virus strains used (WSE, N-M, NWS) have the capacity, on chorioallantoic inoculation into the fertile egg, to kill the chick embryo within 4 days with the production of haemorrhagic encephalitis and multiple haemorrhages in the muscles and lungs. This character was designated e. Eggs inoculated with WSM, N-WSM and WS−, on the other hand, usually survive the 4-day incubation period or, if death occurs, no haemorrhagic lesions can be detected in the embryo. This negative character was designated E.

The following technical procedure was followed. The virus inoculum was diluted in HSS to a titre of 1 agglutinating dose (A.D.) and 0·05 ml. placed on the chorioallantois of each of 4 12-day old chick embryos. The eggs were incubated for three days, then candled to detect early deaths and all surviving eggs were incubated for a further day before being opened. The method of scoring the lesions was as follows: Death on the third day scored 2, on the fourth day 1. The degree of haemorrhagic encephalitis was graded 1-3, muscle haemorrhages 1-3 and lung haemorrhages 1-3, making a total possible score of 11. Scores of 0-2 were regarded as non-specific and rated E, scores of 3 or higher were rated e.

Ff. Pathogenicity for mouse lung. The symbol f was given to viruses which, when diluted to a titre of 1 AD and inoculated intranasally into anaesthetized 4-week old mice caused death of the mice with complete lung consolidation within seven days of inoculation. Viruses which, when inoculated under these conditions, failed to kill mice and produced lung lesions covering usually less than half the total lung area were designated F.

Gg. Neuropathogenicity for mice. The viruses NWS, N-M and N-WSM, on intracerebral inoculation into mice, give rise to neurological symptoms which frequently lead to death of the mice. This character is given the symbol g. Its absence as in the viruses WS—, WSE and WSM is indicated by the symbol G.

The following method was used: The virus inoculum was diluted in HSS to a titre of 1 AD and 0·03 of this dilution was inoculated intracerebrally into 4-week old mice under light anaesthesia. The mice were examined daily for 12 days to record development of convulsions and paralysis or deaths. Twirling the mice by the tail proved a useful diagnostic aid. At the end of 12 days the surviving mice were usually discarded.

It was often necessary to identify the infecting virus by re-isolation from the brains of sick or dead mice. In these cases the sick mice were killed and the brain removed, ground with sterile alundum powder and suspended in 2 ml. of saline. The supernatant fluid, after light centrifugation of this suspension, was used as standing material for egg inoculation procedures. When brains were removed from mice already dead, antibiotics (penicillin and streptomycin) were included in the suspending fluid.

It will probably be convenient to tabulate here (Table 2) the composition of the various strains with which we have been concerned in terms of their marker characters.

TABLE 2.

Genetic composition of virus strains used.

Non-neurotropic strains		Neurotropic strains	
WSE	abdf-ceG	NWSE	abdf-ceg
WSM	aB'df-cEG	NWSM	aB'df-cEg
WS–	abdf-CEG		
"Non-neuro NWS"	ab'(d)f-(c)eG or ab'(d)f-(c)EG	NWS	ab'(d)f-(c)eg
MEL	ABDF-CEG	N-MEL (N-M)	ABDF-ceg

Preparation of first cycle fluids in the de-embryonated egg.

The technique of preparing the de-embryonated eggs was adapted from that of Bernkopf (1949), and that of preparing first cycle fluids from the work of Cairns and Edney (1952). Their use in recombination experiments has already been fully described (Burnet and Lind, 1954a). The essentials of the method were to empty the eggs of all but the chorio-allantoic, allantoic and amniotic membranes and, after washing, to inoculate sufficient quantity of a mixture of two viruses, prepared in a suitable diluent, to ensure that a large proportion of the cells would be doubly infected with at least one particle of each virus type. The inoculated eggs were fastened to a revolving disc, the shaft of which rotated at an angle of 15° from the horizontal in a special incubator maintained at 37° C. After time had been allowed for virus absorption to occur, the eggs were treated with RDE to remove virus still present on surface receptors and to remove any virus receptors which remained intact. The eggs were then thoroughly washed out, refilled with a suitable amount of diluent and replaced in the incubator. Haemagglutinin began to appear in the bathing fluid between the third and fourth hours after initial infection and the contents of the eggs were harvested between the sixth and eighth hours. This harvest contained mostly virus which had been liberated after a single growth cycle in the cells of the egg membranes and is referred to as first cycle fluid.

Limit dilution (LD) titration.

Serial dilutions of virus usually at $\log_{10}$, occasionally at 0·5 $\log_{10}$, intervals were prepared in HSS and 0·05 ml. amounts inoculated allantoically into groups of 4 or more 11-day chick embryos. After 2 days' incubation at 35° C. samples of allantoic fluid from each egg were tested for the presence of virus haemagglutinin. Allantoic fluids were harvested from eggs giving positive reactions and negative eggs returned for a further day's incubation before retesting. In these experiments only positive fluids derived from eggs inoculated with the highest infective dilution of virus, and occasionally these from eggs inoculated with the dilution below this, were examined further. These are referred to subsequently as limit dilution or LD fluids.

EXPERIMENTAL.

Three different sets of experiments were carried out, the first using N-M and WS— as parent strains, the second using N-WSM and WSE, and the third using NWS and WSM. These will be described separately.

1. N-M/WS— Recombination Experiment.

The virus strain N-M differs from the M+ used in recent work on M+/WS— recombinations (Lind and Burnet, 1953) only in having neuropathogenicity. It would, therefore, be expected that an N-M/WS— experiment might give rise to neurotropic WSE types.

Since N-M tends to grow more slowly than WS— in the cells of the allantoic cavity, the inoculation of de-embryonated eggs was arranged to give N-M a slight advantage over WS—. Two different methods were used, one giving N-M the advantage of time, the other of dose. A third set in which equal amounts of both viruses were added simultaneously was also included.

Pairs of de-embryonated eggs were inoculated as follows:

(I) 4·5 ml. N-M diluted to a titre of 20 AD followed after 1 hour's incubation by 0·5 ml. WS— (HA titre=200), giving a final WS— titre of 20 AD.

(II) The same procedure was followed except that the dose of WS— followed 30 minutes after the inoculation of N-M.

(III) The same procedure was followed except that WS— was added immediately after N-M.

(IV) 2·5 ml. of N-M (HA titre=40) and 2·5 ml. WS— (HA titre=20) were added simultaneously.

(V) 2·5 ml. of N-M (HA titre=40) and 2·5 ml. WS— (HA titre=10) were added simultaneously.

Subsequent treatment was as described under "Methods," with minor modification where necessary to ensure that each inoculum was allowed at least one hour for attachment to cells before RDE was added.

The contents of the eggs were harvested 8 hours after initial infection and appropriate pairs of fluids were pooled. These first cycle fluids were tested directly in mice by preparing 10^{-2} dilutions in both anti-MEL serum 1/25 and anti-WS serum 1/25. Dilutions in anti-MEL serum were inoculated intracerebrally into groups of 4 mice to detect any WS type strain which had acquired neurotropism (g) and dilutions in anti-WS serum intranasally to detect any MEL type strain which had acquired mouse lung pathogenicity (f). The mice were examined daily for signs of sickness, or death.

Table 3 summarizes the results of these inoculations. The virus strains isolated from the brains of sick and dead mice are also shown.

TABLE 3.

Results of mouse inoculation of first cycle fluids from N-M/WS– experiments.

Route of mouse inoculation	Diluent	First cycle fluid used as inoculum I§	II	III	IV	V
Intranasal	anti-WS serum 1/25	— — — —	— — — —	— — — —	— — — —	— — — —
Intracerebral	anti-MEL serum 1/25	— — — —	7* — — (78311)†	9 (78312)	+sk10 — — — (78319)	sk10 — — — (78327)	sk7 — — — (78335)
Virus isolated from mouse brains			N-WSE	N-WSE	nil	N-M	N-WSE

—=Mouse survived and showed no symptoms during observation period or lesions at autopsy.
7*=Mouse showed neurological symptoms and died 7 days after inoculation.
+sk10=Mouse sick and killed 10 days after inoculation.
(78311)†=Serial No. of mouse.
§=For derivation of inocula I-V, see text.

None of the mice inoculated intranasally died during the 7-day observation period. At the end of this time they were killed and the lungs examined. In no case were any lesions found. It was therefore concluded that no recombinant strain of MEL serological type with a change of character from F to f had been produced in detectable amounts in this experiment.

TABLE 4.

Neuro-WSE strains isolated by mouse inoculation of N-M/WS– first cycle fluids.

No. of mouse from which virus strain isolated	Limit dilution passage No.	Fluid No.	Neuropathogenicity for mice	Genetic composition
78311	1st LD	0246	7*, 8, S, S	abdf-ceg
	2nd LD	0400	6, 7, 8^{w}, 8	abdf-ceg
	3rd LD	1494	S, —, —	abdf-ce(g)
	4th LD	13187	6, 7, —, —	abdf-ceg
78312	1st LD	0356	4, 6^{w}, 6, 6	abdf-ceg
	2nd LD	1352	6, 6, —, —	abdf-ceg
	3rd LD	13179	5^{w}, 6, 6, 6	abdf-ceg
78335	1st LD	0258	6, 6, 6, 8	abdf-ceg
	2nd LD	0411	5, 5, 6^{w}, 7	abdf-ceg
	3rd LD	13208	6, 6, 7^{w}, 7	abdf-ceg
	4th LD	13576	5, 6^{w}, 6, 8	abdf-ceg

7*=Mouse showed neurological symptoms and died 7 days after inoculation.
S=Mouse sick but recovered.
—=Mouse survived and showed no symptoms during observation period or lesions at autopsy.
w=Virus with the *in vitro* character of WSE was isolated from the brains of these mice.

Among the mice inoculated intracerebrally with first cycle fluids and anti-MEL serum five showed nervous symptoms of which two died. From the brains of three of these mice strains of virus with the *in vitro* characters of WSE were obtained by limit dilution titration in eggs. Each of these strains was given at least 3 consecutive limit dilution passages in the allantoic cavity, each of the LD fluids being tested for *in vitro* characters and for neuro-pathogenicity in mice. The results are given in Table 4. In each case the fluid shown was used as starting material for the next limit dilution passage.

Three lines of neuro-WSE (N-WSE) were thus established, the virus in each case having the marker pattern (abdf-ceg). In terms of the symbols for marker characters WS— (abdf-CEG) and N-M (ABDF-ceg) recombine to give N-WSE (abdf-ceg).

These three N-WSE strains were all isolated by intracerebral inoculation of mice with fluids containing the products of one growth cycle of N-M and WS— inoculated in various proportions and at various time intervals into de-embryonated eggs. Anti-MEL serum was included in the inoculum to suppress the growth of the neurotropic parent virus N-M. The possibility, however, remains that the N-WSE strains were actually produced in the mouse brain by some process in which mouse nerve cells played a necessary part. To exclude this possibility it was decided to carry out a second series of isolation procedures in which the same first cycle fluids were diluted in anti-MEL serum and inoculated both into the allantoic cavity and on to the chorioallantois of chick embryos to see if N-WSE could be isolated without the use of mouse tissues at any stage.

The first cycle fluids II and IV, which had been stored at —70° C., were thawed and serial tenfold dilutions were prepared in anti-MEL serum 1/25. These were inoculated allantoically into eggs and a second inoculum of the same two fluids, diluted 10^{-2} in anti-MEL serum 1/25, was placed on the chorioallantois of 12-day chick embryos.

The allantoic titration gave rise to a mixture of virus types, most of them WS—. Only one, fluid 14848, obtained from a 10^{-5} dilution of first cycle fluid IV in anti-MEL serum, had the *in vitro* qualities of WSE. On intracerebral inoculation this fluid gave rise to neurological symptoms in all mice and caused death in 2/4 on the 4th and 8th days. However, on limit dilution passage, it gave rise to WSE fluids with only very limited mouse pathogenicity. Of five LD fluids tested only one caused a single specific death, one caused neither symptoms nor death, while a proportion of the mice inoculated with the other three showed neurological signs but none died.

The eggs inoculated on the chorioallantois gave the results shown in Table 5.

Lungs from all dead embryos were removed, ground with alundum powder and suspended in 2 ml. of saline. The supernatant fluids obtained by spinning these suspensions were tested for haemagglutinin, and if the titre were high enough, anti-haemagglutinin tests were made with suitable immune sera to determine the serological type of the virus present.

TABLE 5.

Results of chorioallantoic inoculation of N-M/WS– first cycle fluids diluted in anti-MEL serum.

	First cycle fluid used as inoculum.											
	I§						IV					
Score of lesions in embryo*	6	0	9	7+ (14860)†	7	6	5	1	0	6	6	7+ (14868)
Serological types of virus present in lung	Mix.		WS	WS		MEL	WS			WS	WS	WS

* For scoring convention, see Methods.
+ Lung suspensions used for subsequent passage.
(14860)†, serial number of egg.
§ For derivation of inocula I and IV see text.

The lung suspensions marked in Table 5 were each given two consecutive limit dilution passages in eggs. In each case the only virus present was found to be of WSE (abdf-ce) character. Representative fluids from each limit dilution passage were inoculated intracerebrally into mice. All were found to be neuropathogenic. The results are given in Table 6.

TABLE 6.

Neuro-WSE strains isolated by chorioallantoic inoculation of N-M/WS– first cycle fluids.

No. of embryo from which virus strain isolated	Limit dilution passage No.	Fluid No.	Neuropathogenicity for mice	Genetic composition
14860	1st LD	15214	7*, 7, 7, S	abdf-ceg
	2nd LD	16159	6, 7w, S, S	abdf-ceg
14868	1st LD	15233	6, 6, 6, 6	abdf-ceg
	2nd LD	16176	6, 7w, 7, 8	abdf-ceg

7*=Mouse showed neurological symptoms and died 7 days after inoculation.
S=Mouse sick but recovered.
w=Virus with the *in vitro* character of WSE was isolated from the brains of these mice.

Neurotropic WSE strains could, therefore, be isolated from the first cycle fluids of this experiment by methods involving only chick embryo inoculation. The marked variation in neuropathogenicity of different fluids will be noticed in the Tables. This will be discussed in the next section. No attempt was made in these experiments to isolate the reciprocal recombinant MEL (ABDF-CEG).

Variations in neuropathogenicity of N-WSE strains.

Among the N-WSE strains isolated by mouse inoculation methods, whose characteristics are detailed in Table 4, there exists a marked variation in neuropathogenic activity. The strain derived from mouse 78335 showed uniform

pathogenicity throughout four limit dilution passages, killing all mice between the 5th and 8th days after inoculation. The strain from mouse 78312 showed a slight fall in activity on second limit passage, which might be due to variations in the susceptibility of individual mice, but third passage material was fully active. The strain from mouse 78311, on the other hand, showed a marked variation in neurotropic character between one passage and the next.

A more detailed study was therefore made with a further limit dilution passage series of this strain (other than that shown in Table 4) using second passage fluid 0400 (see Table 4) as starting material, inoculating a larger number of eggs than before and fully examining all positive fluids obtained with inocula at or near the limit infecting dilution. The results are shown in Table 7 and give additional evidence of this irregularity.

TABLE 7.

Neuropathogenicity of N-WSE strain from mouse 78311.

Inoculum	Dilution inoculated	LD passage No.	Character of LD fluids: Serial No.	Mouse neuropathogenicity	Other characters
0400	10^{-7}	3rd LD	2912	S, S, S, S	abcde
	10^{-8}		2915	6, 6, 6, 6	abcde
			2916	6, 6, 8, S	abcde
			2917	7, —, —, —	abcde
2916	10^{-7}	4th LD	5133	—, —, —, —	abcde
			5135	6, 11, —, —	abcde
			5139	7, 11, —, —	abcde
	10^{-8}		5140	—, —, —, —	abcde
			5141	7, 7, 7, —	abcde
			5142	6, 12, —, —	abcde
			5143	S, —, —, —	abcde
			5144	—, —, —, —	abcde
			5145	—, —, —, —	abcde
			5146	—, —, —, —	abcde

Legend as for Table 4.

This variation in neurotropism will be dealt with in more detail in the discussion.

II. N-WSM/WSE Recombination Experiments.

In a second series of experiments the two viruses N-WSM (aB'df-cEg) and WSE (abdf-ceG) were used. In the first experiment the following virus mixtures were inoculated into pairs of de-embryonated eggs:

(i) 5 ml. of a mixture of N-WSM and WSE viruses adjusted to give a final agglutinin titre of 20 AD of each virus.

(ii) 5 ml. of a mixture of the two viruses adjusted to give a final agglutinin titre of 20 AD of N-WSM and 3 AD of WSE.

The first cycle fluids were harvested 6 hours after initial infection and titrated to limit dilution by allantoic inoculation of chick embryos. After incubation the virus-positive fluids, inoculated with dilutions at or near the limit infecting dilution, were harvested and submitted to the usual marker tests. Two titrations of these were made and LD fluids tested, primarily for heat stability and neuropathogenicity. The great majority of the fluids showed the behaviour of one or the other parent type, but there was a noticeable variability in the neuropathogenicity of the heat stable (N-WSM) types. Only two fluids were obtained which appeared to combine characters derived from both parent strains. From one of these, 7444, derived from (i), the first LD titration gave two types, both of which were further studied. The second, 7449, came from the de-embryonated eggs which had received inoculum (ii).

TABLE 8.

Recombinant strains isolated in first experiment using N-WSM (B'Eg) and WSE (beG) as parent strains.

LD fluid from titration of first cycle pool	Limit dilution passage No.	Serial No. of fluid	Heat resistance	Invasiveness for chick embryo*	Neuropathogenicity for mice	Character
7444			B'	7, 5, 1, 6	—, —, —, —	B'eG
	1st LD+	8552	b	0, 2, 2, 1		bE
	2nd LD+	9370	b	0, 0, 0, 1	—, —, —, —	bEG
	3rd LD	10193	b	2, 1, 1, 1	—, —, —, —	bEG
	4th LD	10940	b	0, 0, 0, 2	—, —, —, —	bEG
	5th LD	12985	b	0, 0, 1, 2	—, —, —, —	bEG
7444			B'	7, 5, 1, 6	—, —, —, —	B'eG
	1st LD+	8558	B'	4, 6, 2, 5	—, —, —, —	B'eG
	2nd LD+	9038	B'	1, 1, 1, 5	—, —, —, —	BeG
	3rd LD+	9317	B'		—, —, —, —	B'
	4th LD§	9974	B'	2, 2, 1, 0	—, —, —, —	B'EG
	5th LD	10179	B'	0, 0, 1, 0	—, —, —, —	B'EG
	6th LD	11248	B'	2, 0, 0, 0	—, —, —, —	B'EG
	7th LD	12997	B'	1, 0, 1, 1	—, —, —, —	B'EG
7449			b	8, 10, 1, 0	5×, 5, 6, 6	beg
	1st LD+	8184	b	5, 1, 9, 5	6, 6, 6, 6	beg
	2nd LD	9352	b	—, —, 0, 3	6, 8, 8, —	beg
	3rd LD	9965	b	2, 4, 2, 3	6†, 6, 6, 7	beg
	4th LD	10898	b	5, 2, 6, 2	3, 6†, 6, 7	beg

+=Other virus strains present at or near limit infecting dilution.

*=Scores of lesions in the chick embryo after chorioallantoic inoculation of virus (see Methods).

§=This limit dilution passage (3rd–4th) was made with dilutions prepared in anti-WSE serum 1/25.

×=Mouse showed neurological symptoms and died 5 days after inoculation.

—=Mouse showed no symptoms throughout observation period.

†=beg type virus isolated from the brain of this mouse.

These recombinant types were taken through a series of limit dilution titrations, after each of which the limit fluids were submitted to appropriate marker tests. As the characters acdf were common to both parents, and therefore to the progeny also, the characters tested for were heat resistance (B'b), invasiveness for the chick embryo (Ee) and neuropathogenicity for the mouse (Gg). Tests for conversion to indicator (Cc and Dd) were included from time to time to ensure that no deviation from the normal had occurred with regard to these characters.

The results of the limit dilution titrations of the first cycle fluids are shown in Table 8.

It will be noticed that a number of limit dilution passages were needed to purify these recombinant lines satisfactorily. As both parent strains were of the same serological type it was not possible to make use of a suitable immune serum to suppress the growth of strains of one type, as was the case in the N-M/WS— series of experiments.

It was found, however, that serum prepared against WSE was less effective against heat resistant B' forms than against the b heat labile forms, and use was made of this fact (see Table 8).

In a second experiment the virus inoculum for the de-embryonated eggs contained N-WSM and WSE viruses diluted in 5 ml. to give a final HA titre of 20 for each virus. The first cycle fluid obtained after the usual sequence of manipulations gave on titration two LD fluids, 8822 and 8827, each with the character beg. These two strains were taken through three further limit dilution passages. One strain (8822) retained its mouse neuropathogenicity for a further passage then yielded virus of beG, i.e., WSE type, and it seems probable that in the first two passages the reactions were due to a mixture. The second strain (8827) retained its beg character through three further limit dilution passages and virus of beg character was isolated from the brains of mice infected with limit dilution fluids of the second and third passage. Table 9 shows the history of this strain.

TABLE 9.

Recombinant strain isolated in second experiment using N-WSM and WSE parent viruses.

	Limit dilution passage No.	Serial No. of fluid	Heat resistance	Invasiveness for chick embryo*	Neuropathogenicity for mice	Character
8827			b	5, 6, 4, 5	5*, 6†, 6, 6	beg
	1st LD	9569	b	7, 5, 7	5, 6, 6, 6	beg
	2nd LD	10604	b	7, 8, 7, 7	6†, 7, 8, 5	beg
	3rd LD	10915	b	5, 4, 6, 4	6, 6†, S, —	beg

S=Mouse showed neurological symptoms but recovered.

Legend otherwise as for Table 8.

Three types of recombinants were thus isolated in this series of experiments: (a) bEG (abdf-cEG), WSE type which is not invasive for the chick embryo; (b) B'EG (aB'df-cEG), N-WSM, which has lost its neurotropism for mice and is therefore similar to standard WSM; (c) two strains of beg (abdf-ceg) or neurotropic WSE.

Comparison of the N-WSE strains obtained in the two sets of experiments.

Representative fluids 10898 and 10915 (see Tables 8 and 9) of the N-WSE (abdf-ceg) strains, obtained by recombination between N-WSM and WSE, were compared in detail with those derived from recombination experiments between WS— and N-M. As the experiments in the latter group had been carried out some time previously, glycerolated samples of fluids 0400 and 0411 (see Table 4), which had been preserved at —12° C., were each given one limit dilution titration. Fluid 0400, at 10^{-6} dilution, yielded two limit positive fluids, 10617 and 10619 and fluid 0411, at the same dilution, yielded 10627 and 10629. Fluids 16159 and 16176 (see Table 6), isolated by egg isolation from N-M/WS— recombination experiments, were also included. The results are given in Table 10.

TABLE 10.

Comparison of characters of N-WSE strains obtained in the two sets of experiments with one another and with the parent strains.

Origin of virus fluid	Virus fluid	b'bBB HA after heating 30 min. at 55° C. Orig.	55° C.	60° C.	65° C.	eE Invasiveness for chick embryo*	gG Neuropathogenicity for mice+	Genetic composition
From N-M/WS— expts.	10617	60	25	<1		4, 0, 6, 5	6, 6, S, S	abdf-ceg
	10619	110	45	<1		7, 6, 2, 3	7, S, S, S	abdf-ceg
(Mouse isolation)	10627	1300	160	<1		7, 4, 0, 6	6, 6, 7, 7	abdf-ceg
	10629	1400	100	<1		1, 3, 1, 5	6, 6, 6, 7	abdf-ceg
(Egg isolation)	16159	80	60	<1		8, 5, 7, 2	6, 7, S, S	abdf-ceg
	16176	160	60	<1		3, 4, 7, 5	6, 7, 7, 8	abdf-ceg
From N-WSM/WSE expts.	10898	450	100	<1		5, 2, 6, 2	3, 6, 6, 7	abdf-ceg
	10915	160	80	<1		5, 4, 6, 4	6, 6, S, —	abdf-ceg
Stock virus	N-M	900	200	80	<1	4, 3, 5, 7	6, 6, 6, 7	ABDF-ceg
	WS—	600	320	<1		0, 0, 1, 0	—, —, —, —	abdf-CEG
	WSE	640	40	<1		7, 7, 9, 9	—, —, —, —	abdf-ceG
	N-WSM	240	80	80	50	0, 2, 0, 0	7, 7, 7, 7	aB'df-cEg

* For scoring convention for chick embryo lesions see Methods.

\+ For scoring convention see Table 4.

Note. All the N-WSE fluids shown were tested for characters Aa, Cc and Dd with completely consistent adc results.

There was thus no significant difference between recombinants obtained by the different methods, and the distribution of neuropathogenicity amongst N-WSE strains can be considered over the whole of the two series. The important feature is the appearance amongst the recombinant strains of clones of lower pathogenicity than are ever obtained from the neurotropic parent alone. In all experiments there are recombinants which allow the survival of some or all of the mice inoculated with the standard dose. Both the neurotropic parents N-MEL and N-WSM were always lethal though some deaths were delayed to the 8th or 9th day in contrast to the almost invariable death by the 6th day with NWS. As has been the case with the other systems we have investigated, repeated LD passage, with tests of a number of LD fluids from each passage, will usually show a downward drift in the average degree of neuropathogenicity.

III. NWS/WSM Recombination Experiments.

The virus strains NWS (ab'(d)f-(c)eg) and WSM (aB'df-cEG) have previously been shown to give rise to the recombinant type neuro WSM (aB'df-cEg) when grown together in mouse brain (Burnet and Lind, 1951). It was therefore of considerable interest to see if similar results could be obtained by recombination in the cells of the allantoic cavity of chick embryos.

TABLE 11.

Character of recombinant strains obtained in NWS/WSM experiments.

Limit dilution passage No.	Serial No. of fluid	Heat resistance	Invasiveness for chick embryo*	Neuropathogenicity for mice	Genetic character
	10277	b'	5, 3, 3, 2	—, —, —, —	b'eG
1st LD	11293	b'	0, 0, 0, 0	—, —, —, —	b'EG
2nd LD	11673	b'	0, 2, 1, 0	—, —, —, —	b'EG
3rd LD	12923	b'	0, 0, 0, 0	—, —, —, —	b'EG
	13469	b'	0, 10, —, —	—, —, —, —	b'eG
1st LD	14987	b'	3, 8, 0, 0	—, —, —, —	b'eG
2nd LD	19490	b'	0, 2, 2, 0	—, —, —, —	b'EG
3rd LD	19597	b'	0, 0, 1, 0	—, —, —, —	b'EG

*=For scoring convention for chick embryo lesions see Methods.

—=Mouse showed no symptoms during observation period.

Each of the fluids shown was used as starting material for the next limit dilution passage.

De-embryonated eggs were inoculated with 5 ml. of a mixture of the two viruses containing NWS at a final titre of 50 AD and WSM at a final titre of 10 AD. The standard procedure was followed and the first cycle fluids were harvested 6 hours after initial infection. Limit dilution fluids obtained by allantoic titration of the first cycle fluid pool in eggs were submitted to the marker tests for heat resistance (b'B'), chick embryo invasiveness (eE) and mouse neuropathogenicity (gG). All limit fluids obtained in this titration were found to be

of WSM (B'EG) character except for one, 10277, which had the character b'eG. This fluid was taken through three further limit dilution passages. The first passage yielded heat labile, non-neurotropic virus which, however, was not invasive for the chick embryo, and this character, b'EG, persisted through the subsequent passages.

In a second experiment the same proportions of both parent viruses were used as before but NWS was inoculated 1 hour before WSM. Limit dilution titration of the first cycle fluids yielded no neurotropic WSM (B'Eg) strains, but a virus of the character b'eG was isolated, represented by the limit fluid 13469.

This strain retained its chick embryo pathogenicity (e) character for a subsequent passage, but further passage yielded only virus of b'EG type as described for the previous strain from 10277. A summary of the characteristics of these two strains is given in Table 11.

DISCUSSION.

The main impression we received from this work after experience with the MEL/WSE system was of the much smaller yield of recombinants. As a rule straight-forward titration of first cycle fluids failed to give any types other than the originals in true LD fluids. Secondary manipulations, which included an element of selection, were almost always needed. In the case of the N-WSM/WSE interaction the recombinants were obtained from fluids at one dilution below the limit of infectivity, and may have been the result of complex secondary recombination in the membranes of the embryo used for titration rather than in the original de-embryonated egg. In one way or another several types of recombinant were obtained and the most reasonable interpretation of our results is that in cells doubly infected with the strains that we have studied only a small proportion of viable recombinants are produced.

In discussing the origin of the types obtained, it is convenient to consider only the three qualities with which we were mainly concerned, heat stability of haemagglutinin (b'bBB'), pathogenicity for chick embryo (eE), and neuropathogenicity for the mouse (gG).

(i) In view of the behaviour of M+/WS— the only reasonable way in which the N-M/WE— reaction can be formulated is:

B-eg/b-EG→b-eg.

where B stands for the whole linkage group ABDF and eg for the other linkage group -ceg.

(ii) The situation with N-WSM/WSE is more complex and it is convenient to look at the probabilities (a) of interchange of whole linkage groups, (b) of reactions involving the more labile virulence genes Ee and Gg only.

On (a) B'-Eg/b-eG could give B'-eG, which was possibly obtained as an unstable form, and b-Eg, which was not isolated in our experiments.

If interchange of g and G only occurred the possibilities are B'E-G and be-g, both of which were isolated in pure stable form.

If in the two types theoretically obtainable under (a), a loss of one or other type of virulence gene occurred, B'-eG might become B'-EG and b-Eg change to b-EG. Both of these were isolated and, in the case of the first, the evidence from Table 8 is quite strong that this is in fact the way the strain was obtained.

(iii) Interaction between NWS and WSM would be expected to give the same N-WSM as we obtained earlier in mouse brain experiments, and in addition a non-neuropathogenic strain with the other qualities of NWS.

B'EG/b'eg→B'Eg and b'eG.

No N-WSM's were obtained, but in two such experiments the expected reciprocal type, non-neuropathogenic but with the characteristic extreme heat lability of NWS, was obtained.

This b'eG form on passage lost its pathogenicity for the chick embryo becoming b'EG. This is probably an example of the phenomenon by which newly formed recombinants have a tendency to lose much of their extrinsic virulence on a single LD passage in the allantoic cavity.

The distribution of the character neuropathogenicity was entirely comparable to what was found in earlier work, the significance of which has been recently discussed (Burnet and Lind, 1954b). The stock strain NWS, which in the last analysis supplied all the neuropathogenicity, killed all mice by the 6th day. The N-M strain used had been chosen for stabilized neuropathogenicity and no survivals beyond the 7th day are recorded, while stock N-WSM had a sharp peak of mortality on the 7th day. Both killed all mice given the standard dose. If we combine all titrations of N-WSE strains with the exception of the 10 LD fluids from a single strain, shown in Table 7, we obtain a distribution with a peak of deaths at 6 days and 24 p.c. of survivors, 14 p.c. of whom had shown neurological symptoms. The lability of the character is also shown well in the results tabulated in Table 7, but this cannot be regarded as a completely typical result.

SUMMARY.

A re-examination has been made of recombinations in which neuropathogenicity for mice has been concerned.

Using the symbols previously adopted with the addition of g for neuropathogenicity, and G for its absence, the following interactions have been observed:

ABDF-ceg/abdf-CEG → abdf-ceg
aB'df-cEg /abdf-ceG → abdf-ceg
abdf-cEG
aB'df-cEG
aB'df-cEG/ab'(d)f-(c)eg → ab'(d)f-(c)eG
ab'(d)f-(c)EG

There was a wide variation in the degree of neuropathogenicity shown by the recombinants. Many experiments showed a proportion of non-lethal strains, and there was a general tendency for the average degree of neuropathogenicity to diminish on continued limit dilution passage.

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THE NUCLEIC ACID CONTENT OF INFLUENZA VIRUS

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For a number of reasons, the main one being lack of a suitable host system, few chemical studies on the mode of influenza virus multiplication have been carried out. Of the hypotheses which have been proposed, Hoyle's (1952) is perhaps the most amenable to experimental approach by chemical methods. Briefly, it is claimed that the elementary particle is composed of two soluble portions, a haemagglutinin and a complement fixing (C.F.) antigen. On the basis that it is precipitated by lanthanum salts and its activity is reduced by ribonuclease treatment, the CF antigen, released from the virus particle by ether treatment, was claimed to be nucleo-protein in nature. Ada and Perry (1954) found that the CF antigen, purified from extracts of infected embryo chicken lungs after removal of haemagglutinating virus, contained ribose nucleic acid (R.N.A.). The relationship between the CF antigen and virus particle is now being investigated and the initial stage, a reinvestigation of the virus nucleic acid content, is reported in this paper.

Considerable disagreement exists in the literature concerning the nucleic acid content of influenza virus. Taylor (1944), using a colour reaction to estimate the sugar moiety, found no reaction for ribose but a reaction for deoxypentose which gave values of 2·1 p.c. deoxypentose nucleic acid (D.N.A.) in influenza A, 3·7 p.c. DNA in influenza B, and "+" in Swine influenza virus. Knight, however, isolated the nucleic acid from influenza A and obtained by the Bial and Dische reactions positive evidence for the presence of both RNA and DNA, the presence of the former being confirmed by tests with ribonuclease. He concluded (1947a) that the values for influenza A were 3 p.c. RNA and 2 p.c. DNA. More recently, Graham (1950) analysed defatted influenza virus A (PR8 strain), using the Schmidt and Thannhauser method to differentiate between RNA phosphorus and DNA phosphorus. In this way PR8 was found to contain 4·5 p.c. RNA and 0·3 p.c. DNA. These differences warranted an investigation of the problem, especially as initial results (unpublished) obtained by the present authors varied considerably from those quoted above.

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MATERIALS AND METHODS.

Virus strain. The classical strain PR8 was used. Allantoic fluids were harvested 42 hours after inoculation of 0·05 ml. of 10^{-4} seed into 11-day eggs. Samples for infectivity and haemagglutinin estimation were taken immediately after pooling of the harvested fluids.

Infectivity titration. 10-fold dilutions were made in 10 p.c. horse serum saline and standard volumes of each dilution inoculated allantoically into 10–12-day eggs (usually 10 eggs per group). After three days' incubation at 35° C., the allantoic fluids were harvested, tested for the presence of haemagglutinin and infectivity end points calculated by the method of Reed and Muench (1938).

Haemagglutinin titre. The fluids to be titrated were diluted in two-fold steps in normal saline, an equal volume (0·25 ml.) 1 p.c. fowl red cells added and the cells allowed to settle at room temperature. The titre is expressed as the reciprocal of that dilution of fluid which shows the "+" degree of partial agglutination.

Solvents. Saline —0·9 p.c. (w/v) NaCl. Eluting fluid, 6·2 gm. NaCl, 12 gm. dextrose, 0·2 gm. $CaCl_2$, 0·25 gm. H_2BO_3, 0·01 gm. $Na_2B_4O_7$, $10H_2O$. Made up to 1 litre with distilled water. pH approx. 7·2.

Nucleic acids. For comparative purposes, samples of semi-purified preparations of RNA and DNA were necessary. Yeast nucleic acid was purified by the method of Smith and Markham (1950) and the DNA from thymus nucleic acid by the method of Signer and Schwander (1949). The preparations analysed 8·3 and 7·9 p.c. phosphorus respectively.

Crystalline ribonuclease. This was a preparation purchased from the Worthington Biochemical Laboratory, New Jersey, and stated to contain no protease activity.

Centrifugation. A Spinco model L centrifuge, fitted with a No. 30 rotor, was used. The specifications of this rotor have been given previously (Ada, Perry and Pye, 1953).

Phosphorus estimation. A slight modification of the method of Ernster, Zetterstrom and Lundberg (1950) was used. The sample containing 0·25–5 μg. P (and less than 0·1 gm. NaCl) was heated with 0·5 ml. 10 N H_2SO_4, the digestion being completed with the aid of H_2O_2 (100 vol., M.A.R.). To the digest was added 3 ml. distilled water and 3·0 ml. isobutanol benzene (1:1). 0·5 ml. 10 p.c. (w/v) ammonium molybdate was added, and the mixture shaken for 1 minute. 2·5 ml. of the top layer was transferred to a test tube and 3 ml. of acid ethanol (96·8 ml. ethanol, 3·2 ml. conc. H_2SO_4) added. The colour was developed by the addition of 0·5 ml. reducing agent (0·25 ml. of 10 gm. $SnCl_2.2H_2O$ in 25 ml. conc. HCl, diluted to 50 ml. with 1 N H_2SO_4) and read in a photoelectric colorimeter. Phospholipid values were obtained using a factor of 25, and nucleic acid using a factor 10·3.

Deoxypentose. The method of Ceriotti was used (1952).

Ultraviolet absorption. A Uvispek fitted with 0·5 and 4 cm. cells was employed. Nucleic acid values were determined using a formula E_P=30·98 E/cl (Ogur and Rosen, 1950).

EXPERIMENTAL.

Purification of virus.

Two main methods have previously been employed. Firstly, removal from infected allantoic fluid by adsorption on to red cells followed by elution into a suitable solvent from which it is further purified by differential centrifugation. Secondly, direct purification from infected allantoic fluid by several cycles of differential centrifugation. While neither method is completely satisfactory, the former is to be preferred as it utilizes the enzymic specificity for its substrate. This is partly counteracted by the fact that on a semi-large scale, it

seems impossible to avoid some haemolysis of the red cells. In this investigation only fresh human red cells were used. Any breakdown of avian erythrocytes would liberate DNA into the fluid with the possible danger of contamination of the virus particle. The following procedure was adopted, all steps being carried out at 2–5° C. unless otherwise stated.

Washed packed red cells were added to the cooled allantoic fluid in the proportion of 2·5 ml. per 100 ml. of fluid, haemagglutinin titre=1,000. The suspension was gently stirred for one hour and the agglutinated cells then separated by brief centrifugation. They were washed once with and then suspended in the eluting fluid, the volume used being one-twentieth of the original volume of allantoic fluid. After incubation with occasional stirring for 1½ hours at 37° C., the cells were removed by centrifugation at room temperature. The supernatant was removed and further centrifuged at 5,000 *g.* for 10 minutes. The supernatant from this was removed and centrifuged at 45,000 *g.* for 30 minutes. The sediment was suspended (usually by letting stand overnight) in saline and clarified by centrifugation at 10,000 *g.* for 10–15 minutes.

The sediment so obtained was re-extracted with saline and the extract clarified as before. The pooled supernatants were then subjected to another cycle of differential centrifugation. Aliquots of the final solution were taken for haemagglutinin and infectivity titrations. Yields in terms of haemagglutinin activity recovered averaged 40 p.c., contained in about 0·3 p.c. of the original volume of allantoic fluid.

Purity of virus.

The validity of results obtained by chemical analysis of the virus will ultimately depend upon the degree of purity achieved. Estimates of this can be made by biological and physical criteria. In the first case, the ratio-haemagglutinin units/mg. dry weight of different preparations have been compared. The maximum value obtained was 10×10^4 units/mg. dry weight. Preparation with ratios less than $7\cdot0\times10^4$ were used only for sighting experiments.

Of the physical criteria available, electron microscopy offers the best means of assessing the purity of the preparations. In Fig. 1 is shown a *typical* field of a purified preparation.

There is a considerable amount of aggregation which prevents an accurate estimation of purity. It cannot be categorically stated on the basis of electron microscopy alone to what extent the structures, which are not clearly defined virus particles, are aggregates of contaminating protein or partially disintegrated virus. In either case, these components would not amount to more than 10 p.c. of the total material.

Chemical Analysis of Virus.

Methods of estimating nucleic acids.

Prior to chemical analysis, the virus was precipitated by the addition of an equal volume of ice cold 30 p.c. (w/w) trichloracetic acid (T.C.A.). Flocculation was encouraged by vigorously scraping the side of the container with a glass rod. After standing at 0° C. for approximately 10 minutes, the precipitate

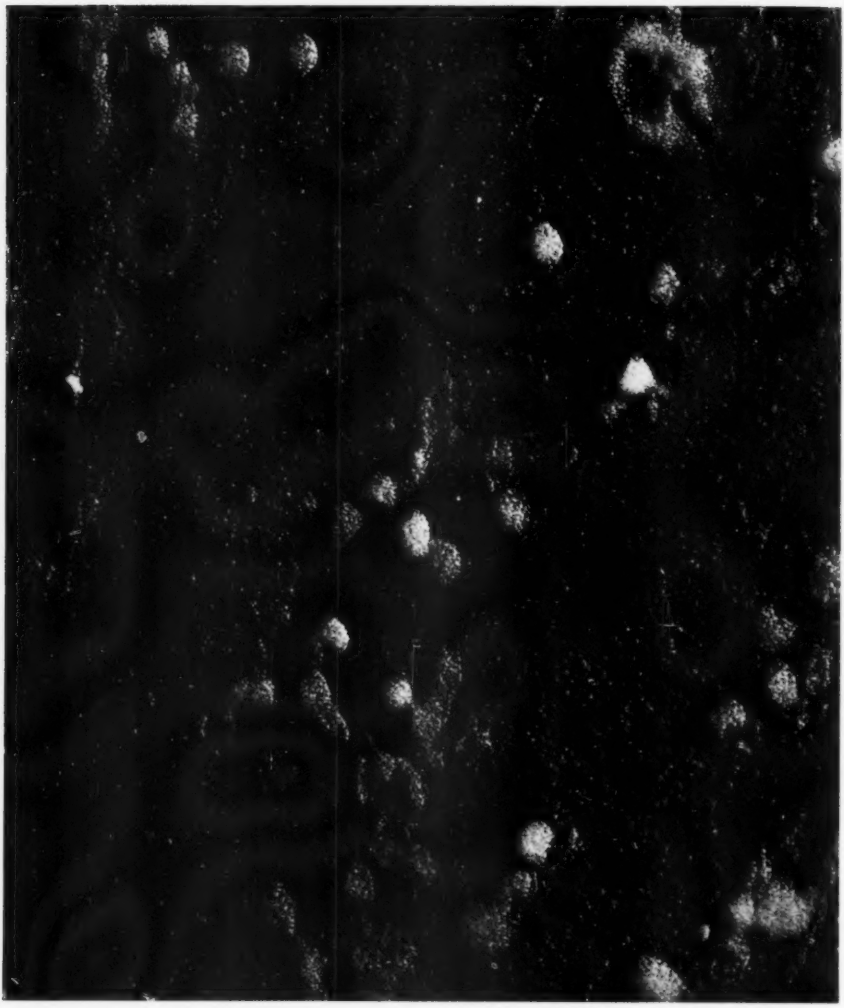

Fig. 1. Electron micrograph of purified influenza virus. Shadowed with gold manganin. Magnification, 65,000 diameters.

was obtained by centrifugation, suspended in a small amount of cold water and dialysed for 16 hours against distilled water. The preparation was then dried from the frozen state *in vacuo*, the final stages being carried out in a vacuum desiccator over P_2O_5.

Nucleic acid may be determined in three ways—by estimating the sugar, phosphate or purine and pyrimidine moieties. Colour reactions for the sugar moieties suffer the major disadvantage that few are specific and all seem to be influenced by the presence of large amounts of protein breakdown products. Furthermore, substances such as polyribophosphates may be classified on the basis of pentose reactions in the RNA fractions (Zamenhof *et al.*, 1953). Ultraviolet (U.V.) determination of the purine and pyrimidine constituents is subject to interference by amino acids such as tyrosine and tryptophane. Technically, phosphorus estimations are very sensitive and the danger of organic compounds interfering in the colour reaction is avoided by the preliminary acid digestion. However, other phosphorus containing compounds, e.g., phospholipid, are present in influenza virus and unless they can be quantitatively determined, the estimations of nucleic acid by phosphorus analysis will be in error. The choice of method is made more difficult in the present case because there is so little nucleic acid present in the virus particle (e.g., Taylor, 1944; Knight, 1947) that the danger of interference by other compounds is heightened. While none of the above methods approaches the ideal, phosphorus estimation was considered the least objectionable and initially was used as the main method. RNA and DNA were estimated using the method of Schmidt and Thannhauser as modified by Davidson *et al.* (1951). The separation is based upon the stability of DNA in the presence of alkali while RNA is hydrolysed to nucleotides.

Effect of solvent used to extract lipid.

In earlier analyses (e.g., Taylor, 1944), phospholipid was found to be the major phosphorus containing component present in influenza virus. As imperfect extraction of this component would result in a high nucleic acid value (as shown by phosphorus remaining in the residue) an examination of the existing methods of extracting lipid was therefore undertaken. The standard procedure involves the use of boiling alcohol, acetone, chloroform and ether. In initial experiments, dried virus preparations were extracted by this method and the residues submitted to fractionation for RNA, DNA and phosphoprotein. The nucleic acid values obtained are given in Table 1 and agree quite well with those obtained by Graham (1950) using a similar process. Morton (1950) has shown that n-butanol acts as a specific solvent for phospholipid, and in two experiments extraction of dried virus with successive portions of butanol at room temperature, followed by washings with ether, was carried out. The results of one experiment are given in Table 1. The third system investigated was a chloroform methanol mixture in the proportion 2 : 1. This was used by Folch and Lees (1951) to extract proteolipid from brain tissue. The results of an experiment in which this solvent system, followed by ether washings, was used is also given in Table 1.

TABLE 1.

Influence of lipid solvent used on the amount of phosphorus left in residue to be estimated as nucleic acid.

Solvents	RNA p.c.	DNA p.c.
Alcohol, acetone, chloroform and ether	6·0	0·2
Butanol and ether	2·12	0·06
Chloroform methanol and ether	1·0	0·04

Comparison of the results clearly shows that the last mentioned system gives the lowest nucleic acid values. The possibility of nucleic acid extraction by chloroform methanol was investigated, but exhaustive tests by ultraviolet absorption techniques failed to reveal any sign of nucleic acid in the extract.

Analysis of phosphorus containing fractions present in purified virus.

As a routine procedure, dried virus (usually 20–40 mg.) has been extracted twice by chloroform methanol, followed by one n-butanol extraction and then two washes with redistilled ether. Extraction was carried out at room temperature over a period of 8 hours, the residues in each case being removed by centrifugation. The final residue is weighed and the difference in weight between original and the extracted material, after correcting in a manner to be described later, regarded as the lipid content. Phosphorus content was determined on an aliquot of the extract and converted to phospholipid using a factor of 25. The proteolipid was isolated from the rest of the extract (vide Folch and Lees, 1951) and weighed. The residue after lipid extraction was analysed for RNA phosphorus, DNA phosphorus and phosphoprotein phosphorus. Details of the technique used have been published previously (Ada and Perry, 1954).

TABLE 2.

Mean values of phosphorus-containing fractions present in purified virus.

Expressed as percentage of whole virus.

Total lipid	Proteo-lipid	Total phospho-lipid	Total lipid* (corrected)	Phospho-protein phosphorus	RNA	DNA
44·0 (9) ±3·8	13·0 (4) ±8·4	10·1 (4) ±0·5	37·5	0·012 (7) ±0·01	1·19 (9) ±0·21	0·053 (9) ±0·037

* See text for explanation. Figures in brackets refer to number of estimations.

The mean values obtained from a series of analyses are presented in Table 2, the figure in brackets in each column indicating the number of analyses averaged. The first three columns deal with lipid components. While the proteolipid component varies considerably in amount, total lipid and phospholipid contents are

relatively constant. In the fourth column is given a figure for corrected total lipid. This has been obtained by subtracting from the value, in column 1, half the proteolipid value in column 2 on the basis that only half the proteolipid complex is chemically lipid (Folch and Lees, 1951).

The phosphoprotein phosphorus values in column 5 show a very large variation. This may be because it is probably a very complex fraction, phosphoprotein phosphorus being merely a convenient title. The RNA figures in column 6 are fairly constant, while the DNA values in the last column show considerable variation.

Though these nucleic acid values are considerably lower than those reported in the literature, the possibility exists that they are greater than the true values. In their work on liver extracts, Davidson and Smellie (1952) have found that 25 p.c. of the phosphorus present in the alkaline digest, obtained by their modification of the Schmidt and Thannhauser procedure, is accounted for by organic phosphates other than nucleic acid. Furthermore, the DNA values for the virus are so small and variable as to suggest that the DNA present is a contaminant, e.g., less than one molecule per virus particle, or indeed may be absent, the phosphorus being other than DNA phosphorus. For this reason an alternative method of estimating the nucleic acid was sought.

Use of the Ogur-Rosen method of estimating nucleic acid phosphorus.

Ogur and Rosen (1950) have described a method, originally designed for plant extracts, whereby the RNA and DNA may be extracted by perchloric acid ($HClO_4$) at different temperatures. Briefly, it was found that, in defatted plant tissue preparations, extraction with 1 N $HClO_4$ at 4° C. overnight, followed by two brief washings, removed all the RNA. DNA was removed from the residue by two extractions with 0·5 N $HClO_4$ at 70° C. for 20 minutes, phosphoprotein phosphorus remaining in the final residue. With animal tissue preparations, it was found necessary to use 1 N $HClO_4$ and heat at 80° C. for 30 minutes to extract all the DNA.

This procedure was followed in several experiments. After lipid extraction, the dried virus material (10–20 mg.) was suspended in 2 ml. of cold N $HClO_4$. After thorough mixing, the suspension was kept overnight at 4° C. and centrifuged next morning. The residue was briefly re-extracted twice more with 2 ml. aliquots of cold N $HClO_4$. The supernatants were pooled and called the RNA fraction. The sediments were extracted twice with 2 ml. aliquots of N $HClO_4$ at 80° C. for 30 minutes. The supernatants were kept separate and called DNA fractions 1 and 2 respectively. UV analysis for nucleic acids was carried out on the isolated fractions, the value, at 260 mμ in the case of RNA and 268 mμ in the case of DNA, being inserted in the equation given in "Materials and Methods." The extracts were then carefully neutralized by the addition of KOH, aliquots of the supernatant being taken for phosphorus and for deoxypentose estimations.

In Table 3 are given the results obtained from one of three such experiments. In this case, the lipids had been extracted with butanol. The residue was divided

into two equal portions, one being fractionated by the Schmidt and Thannhauser (S-T) procedure and the other by the Ogur-Rosen (O-R) procedure. It is clear that there is a striking difference in the results obtained by the two methods. The RNA value obtained by both UV and phosphorus determination in the O-R analysis is substantially lower than the value obtained by phosphorus determination in the S-T procedure. On the other hand, the DNA value obtained by phosphorus estimation in the O-R analysis is very much higher than either the UV and sugar results on the same sample or the phosphorus value obtained by the S-T procedure. This could only indicate that these were phosphorus-containing compounds other than nucleic acids, which in the S-T method contributed to the RNA fraction and to the DNA fraction in the O-R procedure. In the first hot $HClO_4$ extract, the UV curve showed a peak at 265 mμ, which is between that given by RNA in cold $HClO_4$ (260 mμ) and DNA in hot $HClO_4$ (268 mμ). Thus it was not clear whether the UV absorbing material in this extract was DNA, RNA or both. The UV curve of the second hot $HClO_4$ extract showed a definite peak at 275 mμ, which is close to that for tyrosine. It is doubtful then whether there was any DNA in the second extract.

TABLE 3.

Comparison of Schmidt and Thannhauser with Ogur and Rosen techniques for the estimation of nucleic acid fractions in defatted virus.

Method	Phospho-protein phosphorus	RNA		DNA 1st extract			DNA 2nd extract		
		P.	U.V.	P.	U.V.	Sugar	P.	U.V.	Sugar
Schmidt and Thannhauser	0·033	1·71		0·01					
Ogur and Rosen	0·008	0·95	0·87	0·80	0·15*	0·13	0·30	†	0·10

Fractions estimated by phosphorus, ultraviolet absorption and sugar determination, the figures being expressed as percentages.

* Peak at 265 mμ. † Peak at 275 mμ.

The O-R method, as such, appeared to offer no improvement on the S-T procedure. If quantitative extraction of the RNA with cold $HClO_4$ could be achieved however, it seemed possible that combining the two procedures would yield a technique in which the nucleic acids could be obtained in fractions freed of other phosphorus-containing compounds. The greatest difficulty appeared to be the complete "wetting" of the dried virus preparation by the cold $HClO_4$. Of several compounds tried, methanol was finally used as a wetting agent. In addition, it was considered that the UV absorption results could be made more quantitative if a suitable protein blank could be obtained as influenza virus is known to contain aromatic amino acids (Knight, 1947b). The ideal for this purpose would be influenza virus containing no nucleic acid. Human serum proteins were considered to be the closest to such an ideal. To the present

authors' knowledge, no mention of the presence of significant amounts of nucleic acids in plasma proteins has been made following the extensive investigations of E. J. Cohn and associates at Harvard. In addition, the present work demonstrates the absence of nucleic acids in serum. Their use as a protein control also has the advantage that, being a mixture of proteins, they would analyse to give a "mean" amino acid content. Therefore, a sample of serum was carefully removed from some freshly-taken blood, the proteins precipitated, washed with TCA in the usual fashion and dried from the frozen state. They were then extracted with chloroform methanol and butanol, washed twice with ether, dried and stored in a desiccator. In each experiment an amount of this preparation, equal to the weight of extracted virus, has been taken and treated in the same way as the virus.

The use of a combined fractionation method.

The following is a description of an experiment in which the O-R and S-T methods were combined. Approximately 20 mg. of defatted virus and serum protein were moistened with a few drops of methanol (A.R.) and vigorously suspended in 5 ml. of cold N $HClO_4$. After storage overnight at 2° C., they were resuspended and centrifuged. The supernatants were removed and the sediments washed with 3 lots of 2·5 ml. cold N $HClO_4$. The supernatants were pooled and called RNA fractions. The sediments were incubated overnight with 3 ml. of N KOH. Next morning, sufficient cold N $HClO_4$ was added to the cooled extract to bring the pH to 1. After standing at 0° C. for 30 minutes, the solution was centrifuged, the supernatant removed, and the sediments washed with cold N $HClO_4$. The supernatants were pooled and labelled alkaline digest. The sediments were extracted with 4 ml. of N $HClO_4$ at 80° C. for 30 minutes, centrifuged, the supernatants removed and the sediments washed with 2 ml. aliquots of N $HClO_4$. After centrifugation, the corresponding supernatants were pooled and called DNA fractions. In this way it was hoped to isolate the non-nucleic acid phosphorus containing compounds in the alkaline digest. It was assumed that pretreatment with cold 1 N $HClO_4$ would not render any DNA present more susceptible to alkaline hydrolysis. As previously, UV determinations were carried out on the extract as such, and phosphorus and sugar estimations on aliquots after neutralization of the $HClO_4$.

TABLE 4.

Nucleic acid analysis of solvent extracted virus and human serum protein using a technique obtained by combining the procedure of Schmidt and Thannhauser with that of Ogur and Rosen.

	RNA extract		Alkaline digest			DNA extract		
Sample	P	U.V.	P.	U.V.	Sugar (DNA)	P.	U.V.	Sugar
Human serum protein	0·10	peak at 275 mμ	0·024*	peak at 275 mμ	0·17	nil	peak at 275 mμ	nil
Virus	0·53	0·56	0·62*	0·11	0·05	0·06	peak at 275 mμ	0·04
Salt extracted virus	0·028	0·01	0·38*	nil	0·05	nil	peak at 275 mμ	0·04

Fractions estimated by phosphorus, ultraviolet absorption and sugar (indole reaction for DNA), determinations, the figures being expressed as percentages.

* Calculated as being nucleic acid.

In Table 4 are given the results obtained from the above experiments and in Figs. 2a, b and c are plotted the UV absorption curves obtained from the three fractions. In both the Table and Figs. are also presented the results of experiments to be described later. In Fig. 2a, Curve 1, is given the absorption curve of the cold $HClO_4$ extract obtained after removal of the control serum blank. The latter amounted to a relatively small adjustment, indicating that little protein is extracted. The resulting RNA curve has a sharp peak between 260–265 mμ. In Fig. 2b, Curve 1, is presented the curve of the alkaline digest obtained after removal of the serum blank. Before adjustment, both virus and serum extracts showed smooth curves rising to a definite maximum at 275 mμ, presumably due to amino acid components. On adjustment however, the virus curve shows a pronounced peak at 260 mμ, indicating the presence of nucleic acid. In Fig. 2c, Curve 1, is given the curve of the hot $HClO_4$ extract obtained after removal of the serum blank. Before this adjustment both virus and serum extracts gave smooth curves rising to a maximum at 275 mμ, and this feature still remained after the adjustment, indicating that there is a negligible amount of nucleic acid present. In the same Fig. is given a curve (No. 3) obtained from a semi-pure preparation of thymus nucleic acid (T.N.A.). The maximum of each of the two curves clearly occurs at different wavelengths.

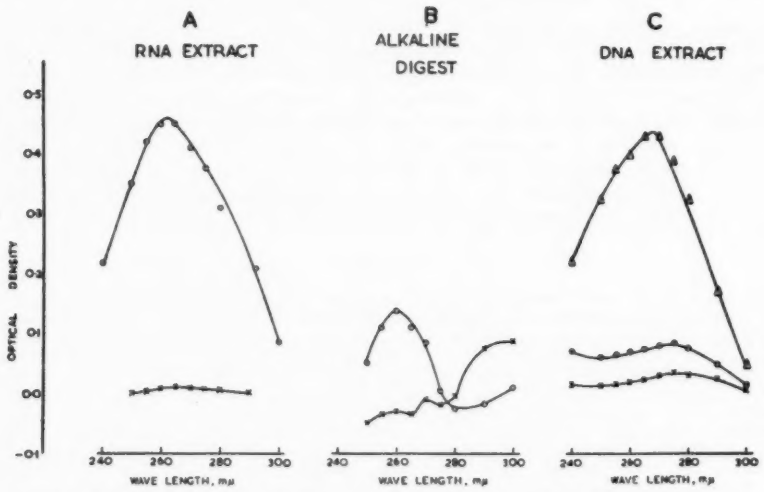

Fig. 2. Ultraviolet absorption curves of extracts of defatted virus fractionated by a combination of the techniques of Schmidt-Thannhauser and Ogur-Rosen. Curves obtained after "correction" with serum protein blank.

Curve 1. O——O Untreated virus.
Curve 2. ×——× Virus extracted with 10 p.c. salt.
Curve 3. Δ——Δ Semi-pure preparation of DNA.

Turning to the overall analyses in Table 4, phosphorus and UV determinations give similar values for the RNA content of the virus. Serum protein shows a significant amount of phosphorus in this fraction, but the UV curve does not show a peak at 260 mμ. In the alkaline digest, the low phosphorus content of the serum extract precludes the presence of any significant amount of nucleic acid although there is a lot of UV absorbing material present. The "corrected" UV analysis of the virus alkaline digest definitely indicates the presence of a small amount of nucleic acid. The phosphorus values allow for a far greater amount than is shown by the UV result, indicating that a large proportion of the non-nucleic acid phosphorus-containing compounds has been obtained in this fraction, as was expected. The phosphorus value in the DNA fraction of the virus is low as in the S-T procedure. The UV curve shows no sign of any nucleic acid being present. Use of the indole reaction for deoxypentose has obviously given inconsistent results, those for serum protein extracts often being as high as those for virus fractions. In all cases, however, the readings obtained were at the bottom of the range where accuracy is least. In separate experiments, not given in detail here, the indole reaction was found to work very satisfactorily using a semi-purified preparation of TNA.

The combination of the two techniques was thus unsatisfactory as a considerable portion of the nucleic acid was extracted in the alkaline digest. Whether this was RNA incompletely extracted by the cold $HClO_4$ or DNA hydrolysed by the alkali was unknown. That it was partly RNA was shown in a separate experiment in which pretreatment of purified virus with ribonuclease reduced the amount of nucleic acid in both the RNA fraction and the alkaline digest. It thus appeared certain that all the RNA is not extracted by the cold $HClO_4$ technique and that at least for this virus the O-R method is unsatisfactory.

Extraction of nucleic acid from virus.

Attention was therefore directed to an alternative method. It was clear that sugar reactions could not be used unless the preparations to be tested were fairly pure. This involved finding a quantitative method of extraction. The method of Schneider (1946), which involves extraction with hot TCA, is quantitative, but apart from the fact that the nucleic acids are degraded in the process, TCA absorb very strongly at 260 mμ, making UV analysis very hazardous. Hot 10 p.c. NaCl has also been used to extract nucleic acids but has been regarded as not quantitative (see Davidson, 1950). However, the degree of extraction seems to have been based on percentage phosphorus extracted from defatted, acid insoluble residues (e.g., Davidson and Smellie, 1952), and the experiments quoted above indicate that by no means all of the phosphorus left behind in this residue is nucleic acid phosphorus.

A sample of defatted virus (from the same batch as above) was thrice extracted with 10 p.c. (w/v) NaCl in a boiling water bath for one hourly

periods. The residue was then subjected to the $HClO_4$—alkaline digest procedure outlined above. The UV absorption curves (corrected) are given in Figs. 2a, b and c, Curves No. 2, and the complete analysis in Table 4. Within experimental error, the absorption curves indicate that the nucleic acid has been completely extracted. The phosphorus analyses in Table 4 indicate that the bulk of the phosphorus-containing non-nucleic acid components have been left behind in the alkaline digest fraction, about 20 p.c. of these components being extracted with the nucleic acid. Thus it appeared that extraction with hot 10 p.c. NaCl could be used, not only to estimate the amount of nucleic acid present, but also to obtain the nucleic acid moiety in a state relatively free of other phosphorus-containing compounds. Tests for the presence of DNA could now be carried out. A sample of the salt extracted virus was submitted to a S-T fractionation. The slight precipitate obtained upon neutralization of the alkaline digest was found to contain no phosphorus, indicating the absence of DNA. A sugar reaction carried out on the salt extract confirmed the absence of any appreciable amount of DNA.

RNA content of influenza virus.

Influenza virus has thus been found to contain only RNA in significant amounts. The actual content was estimated as follows. Three extractions with hot 10 p.c. NaCl were carried out, but it was subsequently found that all the nucleic acid was released into solution after 20 minutes at 100° C. It could then be obtained by centrifuging the suspension and washing the sediment twice with 10 p.c. saline. An equal weight of defatted serum protein was likewise treated and used as a control in UV absorption determinations. The absorption values from 240 mμ to 300 mμ were read, the maximum at 260 mμ being used to calculate the nucleic acid concentration. The mean and standard deviation of nucleic acid content obtained in this way from four different preparations of purified virus were 0·78±0·02 p.c. Determination of phosphorus content gave the following values for the same samples, 0·98±0·08 p.c. The viruses were purified from infected allantoic fluids, the ratios infectivity per haemagglutin unit averaging at 6·0±0·03.

TABLE 5.

Comparison of the U.V. absorption spectra of saline extracts of defatted influenza virus preparations with that of purified yeast nucleic acid.

Sample	260 mμ / 240 mμ	260 mμ / 250 mμ	260 mμ / 280 mμ	260 mμ / 290 mμ
YNA	1·75	1·14	2·08	5·2
Virus extracts	1·54± 0·20	1·14± 0·04	1·94± 0·10	4·5± 0·4

See text for explanation.

The accuracy of the values obtained by UV absorption can be judged by comparing the ratios of optical density at 260 mμ over the values at other wavelengths with those given by a sample of purified yeast nucleic acid. The mean values given by the three extracts quoted above are given in Table 5. The values given by the ratios 260 mμ/250 mμ and 260 mμ/280 mμ are very close to those given by the yeast nucleic acid. The values given by the ratios 260 mμ/240 mμ and 260 mμ/290 mμ are definitely lower than the corresponding values of the yeast nucleic acid, and as these are the more sensitive indication of purity, it seems probable that the values given above for the RNA content of influenza virus are slightly high.

Is the nucleic acid an integral part of the virus?

Despite the low content found, the possibility must be borne in mind that part or all of the nucleic acid is not an integral part of the virus. Two series of experiments were carried out in an attempt to throw some light on these aspects.

Effect of ribonuclease. To 800 ml. of freshly harvested infected allantoic fluid were added 3·2 mg. crystalline ribonuclease (previously dissolved in 2 ml. saline) and the mixture incubated at 37° C. for 2 hours. A control containing no ribonuclease was similarly incubated. Samples were then taken for infectivity and haemagglutinin titrations. The values were found to be unchanged by the treatment. The virus was purified from both samples, and upon estimation, both preparations were found to contain similar amounts of RNA.

Samples of purified virus were also treated with ribonuclease. Short term incubation experiments (1–2 hours) did not reduce the RNA content. Samples were also incubated in the presence of toluene for 16, 24 and 48 hours. In no case did the haemagglutinin titre significantly change. In the 24-hour experiment the RNA content was approximately halved. Unfortunately, the control sample was lost in an experiment in which virus samples were incubated for 16 hours and 48 hours with ribonuclease. These yielded figures of 0·45 and 0·42 p.c. respectively, indicating that probably 30–40 p.c. of the RNA had been removed.

Treatment with cold 10 p.c. NaCl. Extraction of virus in the cold with 10 p.c. NaCl to remove contaminating nucleic acid has been used in the past (e.g., Dawson and McFarlane, 1948). An experiment was carried out in which purified virus was stored in 10 p.c. NaCl at 2° C. for ten days. A control was stored in 1 p.c. NaCl. Both were then rapidly dialysed with stirring against 2 p.c. NaCl and centrifuged at 45,000 *g* for 30 minutes. The deposits were resuspended, precipitated with TCA and analysed as usual. The RNA values were found to be identical.

DISCUSSION.

Influenza virus has been considered unique in that of all the viruses obtained in a relatively pure state, it was believed to contain both RNA and DNA. These

claims were largely based on colorimetric reactions for the sugar moieties, procedures which are reliable when substantial amounts of the sugar concerned are present, but which are unreliable when the sugar to be estimated is present in micro amounts and cannot be satisfactorily separated from protein and polysaccharide degradation products. These latter conditions apply to a marked degree in the case of influenza virus. Unfortunately, analogous limitations apply to the two other techniques available, phosphorus and UV absorption determinations. As far as the determination of virus fractions by phosphorus analyses is concerned, the importance of adequate extraction of lipids has been shown by the results given in Table 1, where a difference of five-fold in the amount of phosphorus left in the residue (estimated as nucleic acid) occurred when different solvents were used. Although more efficient than the other solvents, it is questionable whether the chloroform methanol extracted all the phospholipid present. The nucleic acid results obtained by the application of the Schmidt-Thannhauser procedure to virus (defatted, using chloroform methanol) are approximately 30 p.c. higher than those finally obtained by UV determination of 10 p.c. salt extracts. This figure is close to that obtained by Davidson and Smellie (1952), who found, in work on liver fractions, that approximately 25 p.c. of the material estimated as nucleic acid by determination of phosphorus was, in fact, other organic phosphates. UV absorption has been found to be the most reliable of the three techniques in the present case. As discussed previously, the main limitations of this method are the presence of aromatic amino acids from the material being studied and the possibility of absorbing substances being accidentally derived from the solvents and reagents used. The former could be overcome by using, as a blank, a protein mixture known to contain no nucleic acids. Human serum proteins have been found to be suitable for this purpose, and their use was the main factor in demonstrating that heating with 10 p.c. salt solution quantitatively extracted the nucleic acid. Some protein as well as the nucleic acid was extracted by the salt solution and correction with the serum blank did not account for all the virus protein extracted as shown by the ratios given in Table 5. This is probably due to the presence of some non-heat coagulable protein or other compound associated with the nucleic acid.

Once obtained in a semi-pure state, application of the Schmidt-Thannhauser procedure and the indole reaction for deoxypentose to the nucleic acid extract indicated that a negligible amount of DNA was present. Influenza virus was thus found to contain only RNA. Reasonably consistent results were obtained and indicated a mean value of 0·78 p.c. by UV absorption and 0·98 by phosphorus analysis. These values are possibly slightly high. Though not conclusive, the evidence suggests that the RNA is an integral part of the virus particle. Extended treatment of virus preparations with 10 p.c. NaCl in the cold or short term treatment with ribonuclease did not significantly decrease the amount of

RNA subsequently isolated from the virus. More prolonged exposure to ribonuclease reduced the amount recovered however, but this may have been primarily due to partial disintegration of the particles. The electron micrograph (Fig. 1) suggests that at least some of the particles may break down fairly readily.

Amongst the animal viruses it has been claimed that Eastern equine encephalomyelitis virus (Taylor *et al.*, 1943), louping ill, and probably mouse polio viruses, (see Hyden, 1947) contain only RNA. Only vaccinia and rabbit papilloma viruses have been definitely shown to contain DNA although there is presumptive evidence of its presence in herpes and related viruses. All the plant viruses which have been chemically studied contain only RNA, but as various authors have pointed out, these viruses are probably not by any means representative of the whole range of plant viruses. Containing less than 1 p.c. RNA, influenza appears to have the smallest amount of any of the viruses yet studied in detail. Thus it offers a striking contrast to some of the plant and bacterial viruses which contain more than 30 p.c. nucleic acid. The significance of such a comparison, however, must await further elucidation of the nucleic acid metabolism of the different viruses.

SUMMARY.

Purified influenza virus (PR8 strain) has been analysed for nucleic acid content. It was found, as judged by ultraviolet absorption measurements, that the nucleic acid could be quantitatively extracted from the defatted virus with hot 10 p.c. sodium chloride. Tests for deoxypentose (indole reaction) and for deoxypentose nucleic acid phosphorus (Schmidt and Thannhauser technique) indicated that a negligible amount of deoxypentose nucleic acid was present in the extract. In four preparations in which the infectivity per haemagglutinin unit varied from 6·2 to 5·6, the average ribonucleic content of the virus was found to be 0·78 p.c. by ultraviolet absorption and 0·98 by phosphorus analyses.

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RECOMBINATION WITH TWO INFLUENZA B STRAINS IN THE DE-EMBRYONATED EGG

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In a previous paper (Perry and Burnet, 1953), it was shown that recombination could occur between 2 influenza B strains, LEE and MIL, in the mouse lung. This was a relatively complex situation involving several cycles of virus multiplication in the lung, which provided opportunity for the possible appearance of mutants and for the play of selective processes before the recombinants could be isolated by allantoic inoculation.

With the development of the use of the allantoic cavity (Fraser and Burnet, 1952) and of the de-embryonated egg (Burnet and Lind, 1954) for recombination experiments, it was decided to apply the latter technique to experiments with influenza B viruses. The technical advantages of the de-embryonated egg (DEE) in providing almost the ideal situation for recombination experiments have been discussed by Burnet and Lind.

In the experiments to be reported in this paper, it is shown that recombination occurs with 2 influenza B strains in the DEE, two reciprocal recombinants being isolated. These 2 recombinants, a mouse pathogenic MIL and a non-pathogenic LEE, can recombine in similar experiments to produce the two parent strains, LEE and MIL. Fluids isolated from recombination experiments give a wider range in mouse lung pathogenicity scores than do the stock strains; this applies especially to the recombinant strains which show all grades of mouse lung pathogenicity.

MATERIALS AND METHODS.

Virus strains. The two influenza B strains, LEE and MIL, as in the previous paper (Perry and Burnet, 1953), were used for all experiments. Virus was grown in the allantoic cavity of eleven-day embryonated eggs, and the allantoic fluid harvested two days later.

Haemagglutinin (HA) titrations. Serial doubling dilutions of virus were prepared in 0·25 ml. volumes of saline, and an equal volume of 1 p.c. fowl cells added. After thirty minutes at room temperature, the tests were read, the tube showing the usual + degree of partial agglutination being the end point. The dilution at the end point contains one agglutinating dose (AD) of virus.

Preparation and inoculation of the de-embryonated eggs. The techniques involved have been fully described by Burnet and Lind (1954). Eggs used were from 14 to 16 days of incubation. In these experiments only the chorioallantois was left inside the egg. The membrane was washed thoroughly with warm Ringer solution and the eggs closed with sterile aluminium caps.

All inocula were diluted in standard Ringer solution (NaCl 0·85 gm., KCl 0·025 gm. and $CaCl_2$ 0·03 gm., made up to 100 ml., with distilled water) to which glucose, penicillin and streptomycin had been added to give a final concentration of 0·1 p.c. glucose, 12·5 units penicillin and 70 μg. of streptomycin per ml. This is referred to as GPS Ringer. Infected allantoic fluid virus was diluted in GPS Ringer to give a haemagglutinin titre between 20 and 50, and 5 ml. added to each DEE. The eggs were then placed on a circular rotating disc inside an incubator.

The inoculum was removed after 1–1½ hours and 5 ml. GPS Ringer containing 200 units of RDE per ml. added for one hour, after which time this was replaced by 5 ml. of GPS Ringer. The use of RDE in obtaining first cycle fluids from the DEE has already been discussed by Burnet and Lind. Haemagglutinin production was tested for by placing one drop of the fluid in 0·25 ml. of 1 p.c. fowl cells. Fluids did not become positive earlier than 5 hours after inoculation, and often later. If the fluids were all positive by 5–6 hours they were harvested, if not then the eggs were left overnight in the incubator and the fluids harvested the next morning.

Characterization of fluids. The fluids harvested from the DEEs were pooled, and each pool titrated in eleven-day embryonated eggs. Fluids from the limiting infective dilution which gave positive haemagglutination were tested for the four characters in which LEE and MIL fluids differ (Perry and Burnet, 1953).

Aa, serological character. Two antisera were used; both had been treated with periodate to destroy non-specific inhibitor (Burnet and Lind, 1954).

(1) Rabbit anti-LEE serum (R801). When equal volumes of a 1/2,000 dilution of serum, 1 p.c. human cells and 5 ADs of the fluid to be tested were mixed together at room temperature, LEE type fluids showed no agglutination, while MIL type fluids gave full agglutination.

(2) Ferret anti-MIL serum (F323). When the test above is carried out with a 1/400 dilution of anti-MIL serum, LEE type fluids show full agglutination and MIL type fluids no agglutination.

Bb, heat resistance of haemagglutinin. Allantoic fluids were diluted with an equal volume of saline and heated to 62° C. for 30 minutes. LEE type fluids showed a fall of HA titre to about ¼–½ the original value, while no haemagglutinin could be detected in the MIL type fluids.

Cc, position in receptor gradient. The method is the same as that given in the previous paper (Perry and Burnet, 1953). Human red cells are treated with doubling dilutions of RDE, and the end point is the dilution of RDE which gives cells that are agglutinated to the + level of partial agglutination by 5 ADs of the virus fluid being studied. MIL is the lowest strain in the gradient, and therefore has the lowest titre.

Dd, pathogenicity for mice intranasally. Mice between four and five weeks of age were inoculated intranasally, while under light chloroform-ether anaesthesia, with 0·05 ml. of virus fluid diluted to 1 AD. Mice alive seven days after inoculation were killed and examined for lung lesions. The method of grading lesions is as follows.

5 is complete consolidation with death on the 4th or 5th day after inoculation.

4 is complete consolidation with death on the 6th or 7th day.

3 is approximately ¾, 2 approximately ½, and 1 approximately ¼ of the visible area of the lung surface consolidated, 0·5 and tr refer to smaller areas of consolidation.

Each virus character is represented by a letter. Capital letters denote LEE characters and small letters MIL characters.

LEE=ABCD MIL=abcd.

Intermediate characters are put in brackets, e.g., (B) denotes a heat stability of the haemagglutinin which is less than normal, but is still greater than that possessed by MII fluids.

TABLE 1.

Character of virus fluids isolated from DEEs inoculated with equivalent amounts of LEE and MIL.

Fluid dilution	Proportion of positive fluids	Fluid Nos.	HA titre Orig.	HA titre H. 62° C.	AHA titre Anti LEE R801	AHA titre Anti-MIL F323	Gradient on RDE cells	Mouse lung pathogenicity (M L P)	Formula
	Stock	**LEE**	**960**	**400**	—	††	48	4·0,4·0,4·0,4·0 (4·0)	**ABCD**
	Stock	**MIL B**	**850**	**<20**	††	—	8	0·0,0·0,0·0,0·0 (0·0)	**abcd**
		202733	**30**	**<2**	††	—	8	0·5,1·0,2·5,1·0 (1·25)	**abcD**
		734	**560**	**210**	—	††	32	4·0,4·0,4·0,3·5 (3·9)	**ABCD**
		735	**320**	**28**	—	††	24	5·0,4·0,4·0,3·0 (4·0)	**A(B)CD**
10^{-6}	**7/10**	**736**	**430**	**<2**	††	—	<8	0·5,1·5,0·5,1·5 (1·0)	**abcD**
		737	**210**	**<2**	††	—	<8	2·0,0·0,1·0,1·0 (0·75)	**abc(D)**
		738	**480**	**210**	—	††	27	5·0,4·0,3·5,3·5 (4·0)	**ABCD**
		740	**320**	**<4**	††	—	<8	4·0,4·0,4·0,3·5 (3·9)	**abcD**
10^{-7}	**1/10**	**755**	**240**	**<2**	††	±	<8	0·0,1·0,0·0,0·0 (0·25)	**abcd**

EXPERIMENTAL.

The interaction of equivalent amounts of LEE and MIL in the de-embryonated egg.

De-embryonated eggs inoculated with equal quantities of LEE and MIL and treated as described in the previous section gave rise to fluids, many of which showed a greater or smaller degree of dissociation between serological character and mouse lung virulence, i.e., some fluids serologically MIL had definite pathogenicity for the mouse lung while some fluids serologically LEE had pathogenicity much lower than that of standard LEE fluids. If we adopt the formulae previously used, LEE=ABCD and MIL=abcd, the results gave evidence that in addition to the parent strains, viruses ABCd and abcD could be obtained. Table 1 shows portion of a protocol of one of three satisfactory experiments of this type.

This shows the appearance of 4 MIL fluids with varying degrees of pathogenicity for the mouse lung, but in this experiment no strains of LEE with abnormally low pathogenicity were obtained. Occasional variation in the characters Bb and Cc from those to be expected from the serological type were observed in the primary isolates, but subsequent passage at limit infective dilution (L.D.) gave fluids of normal character in all but one instance. This was a fluid, 03709, with the formula AbCd, i.e., differing from LEE in having no pathogenicity for the mouse lung and possessing heat-labile haemagglutinin. Two successive LD's gave fluids all of which had these characteristics.

Mouse pathogenic MIL strains (abcD) retained their pathogenicity on LD passage with some variation in its intensity as shown in Table 2.

TABLE 2.

Limit dilution passage of mouse pathogenic MIL fluids (abcD).

Fluid No.	M L P	1st LD M L P	2nd LD M L P
95827	2·5	0·9,3·4	2·0,2·5,3·0,2·2 1·5,1·9,1·5
95834	1·1	1·0,0·75,0·7,0·5	
02745	3·2	2·6,2·6,2·6,2·5,1·5	

M L P=Mouse lung pathogenicity as shown by average grading of lesions from an inoculum of 1 AD.

Experiments in which one virus was given an advantage over the other.

Two types of experiments were carried out in which either LEE or MIL was given an advantage in infecting the allantoic cells. In the first type of experiment, one virus was inoculated 30 minutes before the second and a further hour allowed for virus absorption. Two such experiments were done and the results from one are shown in abbreviated form in Table 3. No significant deviations in Bb, Cc characters were observed and only mouse lung pathogenicities are shown.

TABLE 3.

Character of fluids isolated from LEE/MIL experiments in which one virus was inoculated 30 minutes before the other.

Virus type	Stock M L P	LEE 30 min. before MIL No.	Mouse lung path.	MIL 30 min. before LEE No.	Mouse lung path.
ABCD	2·9	8	4·0 3·7,3·6,3·4,3·0 2·4,1·5,1·1	2	3·2,0·4
abcd	0·0	1	0·0	3	0·1,0·0,0·0
ABCd		1	0·0	1	0·1
abcD		1	1·4	1	2·1

Two fluids subsequently shown to be mixtures are omitted.

In the second type of experiment, which was only done once, the two viruses were inoculated simultaneously but the concentration of one was ten times that of the other. The results were very similar to those shown in Table 3, the most interesting feature being the appearance of non-pathogenic LEE strains, ABCd, in both groups. Two examples of ABCd fluids were tested for stability at LD and gave precisely similar reactions, with no mouse lung pathogenicity in any of the 3–4 descendant fluids tested. If we combine all experiments done with MIL/LEE mixtures, the primary isolates comprised 4 types—ABCD (40), abcd (20), ABCd (10), abcD (11), plus 16 ABC fluids with abnormally low MLP but not zero, and 11 abc fluids with low but definite MLP. The significance of these intermediate forms is discussed later.

TABLE 4.

Back-recombination experiments.

Mixture used	Types obtained from first cycle fluids		
ABCd/abcD	ABCD 3 abcd 3	ABC(D) 2 abc(d) 3	ABCd 3 abcD 2
ABCd/aBCD	ABCD 3 aBCD 2	ABCd 2 aBCd 1	

Back recombination of primary recombinants ABCd and abcD.

In a few experiments recombinant strains from either the mouse experiments previously reported (Perry and Burnet, 1953) or the current de-embryonated egg experiments were used after repeated re-isolation at LD had established their stability. The pairs were ABCd x abcD and ABCd x aBCD. These were derived as follows:

abcD, mouse pathogenic MIL from previous DEE experiments.
ABCd, nonpathogenic LEE from previous DEE experiments.
aBCD, MIL serologically, but with all other attributes of LEE from the

experiment described by Perry and Burnet (1953) in which mouse passage of a primary recombinant abcD gave one fluid of this character. Its stability had been established by two further limit dilution passages. The results of these experiments are summarized in Table 4.

Variations in heat stability, Bb.

In general heat stability at 62° C., Bb has been a clear-cut differentiating factor. Of stock LEE strains only 3/16 showed more than two tubes reduction (i.e., to 25 p.c. of original titre), while no stock MIL strain is recorded as ever showing an agglutination. All recombinant LEEs conformed to type, as did all abcD recombinants.

Significant deviations were found with two recombinants.

(1) aBCd from aBCD/ABCd crosses. Substrains from these had a lower average titre after heating and were more variable. Strain 98925 was reduced to <1 p.c., but its descendants gave the following values: 60,40,30,30,30,25,20, 16,15,12,9,8,7,5,4,2,5,<1. Strain 98010 was reduced to 18 p.c. and gave descendants with values as follows: 100,50,45,40,30,30,30,30,25,25,25,16,15,7,6.

(2) In an experiment with ABCd/abcD three recombinants, two acd, one acD, showed distinct resistance to heat with values of 36,6 and 1·5 p.c. On passage, however, all descendants were completely inactivated at 62° C.

There is, therefore, no evidence that heat stability is a quantitative character analogous to mouse lung virulence. Most of the variation is probably due to minor differences in the ionic and protein content of the allantoic fluid. The temporary abCd character is presumably due to some other cause than a variable dosage of the gene in question.

DISCUSSION.

As can be seen very clearly in Tables 1–3, the virulence shown by recombinant strains for the mouse lung varied widely and to some extent the difference between D and d was an arbitrary one, d being taken as representing an average score of 0·25 or less. In the analysis of the results, therefore, particular attention was paid to the distribution of virulence and we have adopted the following arbitrary classification of levels of mouse lung virulence. The lesions were noted in our records according to the usual convention as given under "Materials and Methods." The four groups we adopted were: I, mice showing average lesions graded 2·5 or higher; II, 1·2–2·4; III, 0·3–1·15; and IV, 0–0·25 (i.e., negative, or with mere traces of consolidation). In each instance the grouping was based on the average score of four, occasionally five, mice. In Table 5 we have applied this grouping to a series of tests on stock LEE and MIL strains and to the various types obtained in the recombination experiments.

TABLE 5.

Distribution of mouse lung pathogenicity (M L P) in different virus types.

Origin	Number of strains in each M L P group LEE I	II	III	IV	MIL I	II	III	IV
Stock viruses	14	3	0	0	0	0	1	32
LEE/MIL expts.								
Equivalent	18	5	3	1	4	3	9	5
LEE advantage	20	5	1	5	0	2	0	2
MIL advantage	2	0	2	4	0	2	2	14
Total exptl. fluids	40	10	6	10	4	7	11	21

It can be seen at once that in the strains experimentally derived the degree to which MLP (Dd) is manifested varies much more widely from one fluid to another than is the case with the stock strains. Further, the results given in Table 2 on the MLP of limit dilution fluids from MIL recombinants shows that the MLP of the original recombinant is also manifested with a minor degree of variability downwards by its descendants. Obviously the results cannot be interpreted genetically by the presence of one or another of two alternative alleles. They indicate a high probability that virulence for the mouse lung is a manifestation of the dosage of a certain allele. The experiments available are not extensive enough to allow any useful calculation of the number and distribution of genetic units, but the following hypothetical model gives a distribution not very greatly dissimilar to that actually found.

TABLE 6.

Binomial distribution with varying number of Vs per genome.

Vs per genome	P.c. distribution of M L P grades I	II	III	IV	
0·3	0	0	3	97	cf. stock MIL
1·2	1	8	26	65	cf. exptl. MIL (MIL advantage)
2·4	13	35	34	18	cf. exptl. MIL (equiv.)
3·7	73	24	3	0	cf. exptl. LEE (equiv.)
3·81	82	17	1	0	cf. stock LEE

Values are given to the nearest whole number.

Suppose that mouse lung pathogenicity is determined by the dosage of a gene V which can be present with either ABC or abc in numbers ranging from 0–4. The corresponding grades of virulence are assumed to be VVVV Grade 1, VVV Grade II, VV Grade III, and V or — Grade IV. A binomial distribution with varying average numbers of V per genome would give the type of results shown in Table 6.

These results have a general resemblance to the experimental findings in the relevant parts of Table 5, and no more than this can be expected in view of the complexity of the conditions. It is, of course, a pure guess that 4 genes only are concerned, and the assignment of the levels of M L P to the different numbers of genes is equally arbitrary. An obvious anomaly is that LEE and MIL should contribute an average of about 2·1 Vs per genome while the progeny has an average of about 3·0 Vs per genome. Nevertheless, there is sufficient similarity to make it virtually certain that a process of this general character must be involved although probably modified by factors which distort the simple random distribution of the V units.

The chief implication of this view is that the V units at some stage in the process of virus replication are available for virtually random distribution amongst the maturing genomes.

The differences found with unsymmetrical crosses with MIL or LEE given an advantage are not easy to explain. When MIL is given the advantage the relatively small numbers of LEE strains obtained have a high proportion of non-virulent types with an average of 1·75 Vs in terms of our hypothetical model. If we assume that all LEE strains with low virulence come from cells also infected with at least one particle of MIL, the preponderance of MIL (abc) as against LEE (ABC) strains and the unduly high proportion of non-virulent LEE strains points to interference with the multiplication of both *ABC* and *V* components, the latter being the more strongly inhibited.

When the advantage is with LEE a large proportion of cells produce only typical LEE, but there is still a higher proportion of avirulent LEE strains produced than was found with equivalent mixtures. No explanation can be offered at this time, and more experiments with this system are clearly called for.

In this discussion we have made no mention of the possibility that most virus particles carry more than one set of genetic determinants (Burnet and Lind, 1954). As yet there is no evidence for this in influenza B, but there is equally no evidence to suggest that what seems to be the standard condition in influenza A strains does not hold for B strains. If the diploid condition is standard, then a proportion of particles in the first cycle fluid will be heterozygotes with a constitution, e.g., like ABC-v/abc-vvv. When such a particle initiates infection in a test egg inoculated at limit dilution, a whole series of new recombinations can occur in the primarily infected cell. The virus liberated from that cell will contain several types which will undergo a test for selective survival before the fluid is harvested. We are liable, therefore, to find that the fluid has properties corresponding to that genotype which of all those possible in virtue of the composition of the initial particle, multiplies most rapidly in the allantoic cavity. It is possible that this is responsible for some of the observed results, e.g., that experimental LEE strains show a higher average mouse virulence than experimental MIL strains.

All but one of the recombinants obtained in these experiments were of the type that could be represented by

$$\text{ABC-D/abc-d} \rightarrow \text{ABC-d and abc-D}$$

the manifestations of d and D being subject to the quantitative factors we have just discussed. The exception, a true breeding strain of the constitution AbCd, must have been derived

$$\text{AC-BD/ac-bd} \rightarrow \text{AC-bd}$$

which would indicate the possibility of a second type of break in the genetic sequence.

We have felt that at the present stage it has become necessary to have some working hypothesis of the genetic behaviour of influenza virus B. We have adopted a view in line with the general assumptions of the Delbruck-Visconti theory of bacterial virus genetics but eliminating for the present at least any postulation of mating and cross over. We assume that the three character differences Aa, Bb and Cc each represent the difference between two alleles, the genes being arranged in the order ACB. Attached to Bb is a variable number of genes v, the number of which determines the virulence of the virus for the mouse lung. The complex may be represented as ABC-vvv. In the host cell this complex may replicate as a whole, or it may fracture and the fragments themselves replicate independently. It is further postulated that repair of any fracture may take place whenever conditions are favourable. The points at which there is the greatest frequency of breaking are those linking v's to one another and to B, the only other point at which breaking has been directly demonstrable is between C and B (giving the strain AC-bd), but in view of the appearance in one early experiment of aBCD a break between A and C must be conceivable. Such a hypothesis is out of line with general genetic theory in postulating that repair always gives rise to the same sequence of loci whereas reversals are known to be possible in higher forms. Until much more work has been done this does not seem to be a valid reason for not adopting the simplest possible means of co-ordinating the experimental results.

SUMMARY.

The two influenza virus B strains LEE and MIL can recombine in the deembryonated egg to give two reciprocal recombinants which differ from the parent strains only in mouse lung pathogenicity.

The non-pathogenic LEE recombinant is only obtained readily when one of the parent strains is given an advantage over the other in infecting the egg.

Back recombination experiments give rise to the two original parent strains.

Experimentally-derived LEE and MIL strains show a much greater range in mouse lung virulence than the stock strains.

The significance of the results, especially the quantitative character of mouse lung virulence, is discussed.

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GASTRIC, DUODENAL AND JEJUNAL MOTILITY IN MAN; PHYSIOLOGICAL STUDIES BY BALLOON-KYMOGRAPHY[1]

by RODERICK ANDREW

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The study of gastric and intestinal motility in man and animals by balloon-kymography has a long and fruitful history (Alvarez, 1948). By this means valuable information has been obtained of normal motility and its behaviour in disease and under the influence of drugs, food and emotion. This method of investigation is, however, open to many criticisms. It is uncomfortable and tedious, affecting intestinal behaviour in a number of possible ways by (i) affecting the higher centres; (ii) distension of the gut by the balloons, stimulating stretch receptors; (iii) interference with venous return; (iv) acting as foreign bodies.

Some of these objections apply to any method which has been used, although that of Quigley and Brody (1952) is probably the least objectionable. They have stated that the conventional water manometer has a natural frequency of less than 1 cycle/second and therefore is incapable of measuring rapid changes in pressure. They have claimed that, by using open-end tubes coupled to an optical manometer, frequencies of 25 cycles/second can be recorded, and that, as quite large pressure changes of one second duration can occur, these would escape recognition when conventional apparatus with closed ends is used.

It is impressive that the results observed by many investigators using different methods have been similar. Whatever the effects may be of the artificial stimuli from the use of balloons, results which have been obtained in this study show that it is unlikely that they seriously upset the validity of the experiments. One would expect effects due to the method used, and operating throughout a given experiment, to influence the result in a regular way and to show up, by inhibition or increasing augmentation, as the record proceeds. But it has been repeatedly observed that periods of quiescence and activity occur at any time in the record, some showing marked activity at the beginning, others only towards the end. The variety of patterns recorded, and their great complexity, are in themselves indications that the gut is behaving, to some extent anyhow, untrammelled by the harness applied.

[1]Part of this work was carried out during the tenure of the "Sol Green Research Scholarship," Alfred Hospital, Melbourne.

In this paper records from balloon-kymography are presented, qualitative factors are in the main studied, and broad patterns of motility behaviour sought from the tracings.

METHOD.

A four-lumen Miller-Abbott tube is fitted with three balloons in tandem, each opening into one channel, the fourth being available for the injection of fluids (Fig. 1). The distances between balloons are shown in Fig. 2. The balloons are of thin latex rubber (condoms). Each lumen leads to a tambor covered with latex rubber and, by a system of levers, pressure changes are recorded on smoked paper. *In all tracings, which are recorded simultaneously, the upper record is from the gastric antrum, the middle record is from the duodenal cap, and the lower from the upper jejunum.* A water manometer forms part of each system. An electrical timer records every 30 seconds.

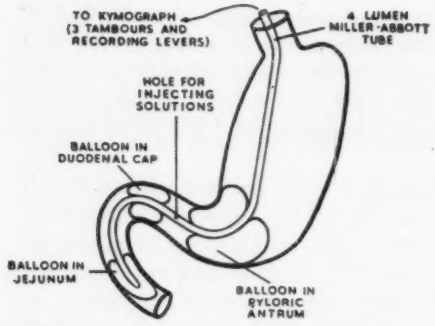
TO KYMOGRAPH (3 TAMBOURS AND RECORDING LEVERS)
4 LUMEN MILLER-ABBOTT TUBE
HOLE FOR INJECTING SOLUTIONS
BALLOON IN DUODENAL CAP
BALLOON IN JEJUNUM
BALLOON IN PYLORIC ANTRUM

Fig. 1. Diagram of 4 lumen Miller-Abbott tube showing positions of 3 tandem balloons.

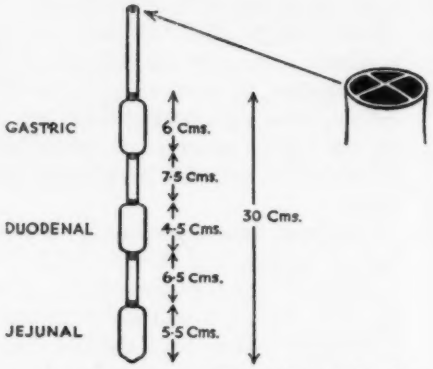

Fig. 2. 4 lumen Miller-Abbott tube showing linear relations between gastric, duodenal and jejunal balloons.

The subjects were healthy medical students, free from gastro-intestinal symptoms. Seven tracings from six students were studied. They fasted for 12 hours before the Miller-Abbott tube, with its attached balloons, was passed at about 9 a.m. When possible this was inserted through the nostril or swallowed if this manoeuvre failed. The subject then lay on his right side and the tube passed through the pylorus with X-ray screen control. Except in the stomach, which initially is usually distended with air swallowed during the passage of the tube, the air-filled balloons show clearly in the X-ray screen. As a rule the distal (jejunum) balloon was through in ½-¾ hour, occasionally passing in a few minutes. When the pylorus was not passed in an hour the test was abandoned. In some instances where the distal balloon was reluctant to move past the pyloric region, gentle pressure along the length of the tube appeared successful. In a few cases, both in this and in other investigations, passage of the balloon through the pylorus appeared to be helped by discussing with the subject his favourite meat and drinks.

It was usually apparent when the pylorus was passed since test inflation led to rapid transit of the distal balloon, clearly seen in the screen, to the third part of the duodenum or, without pause, to the jejunum at the level of the ligament of Treitz. At this stage the gastric

balloon was inflated to prevent its passage through the pylorus, the distal balloon coming to rest in the proximal part of the jejunum and anchored there by the proximal balloon in the gastric antrum. The distances arranged between balloons (Fig. 2) meant that the middle balloon was in the duodenal cap. (Earlier work with a tube fitted with only two balloons —gastric and duodenal—had shown how hard it was to keep the distal balloon in the cap. In the present arrangement, the jejunal balloon acts as a kind of sea-anchor.) The duodenal balloon was then inflated and the tube fixed to the cheek by sticking-plaster. An X-ray photograph at this stage gave a permanent record of the correct placing of the three balloons (Fig. 3).

Fig. 3. X-ray showing positions of gastric duodenal and jejunal balloons on 4 lumen Miller-Abbott tube.

In this control group and in those with peptic ulceration—the subjects of other investigations—it was usually found that rapid distension of the balloon gave rise to vague discomfort, sometimes pain, and nearly always in the mid-epigastric region. This site of reference was the same for distension of any of the three balloons.

These preliminaries over, the subject was wheeled on a trolley to a bed, and the three channels connected to the appropriate tubes on the kymograph. Without allowing any deflation—the pressure in each was adjusted to 8-10 centimetres of water. Recording was then started. About 1-2 hours elapsed between the swallowing of the tube and the start of record. Reassurance was given throughout and relaxation encouraged. Some read a book, others tended to doze although sleep was discouraged. Surprisingly little emotional reaction occurred as the result of this uncomfortable proceeding.

RESULTS.

The results of these investigations are presented under five general headings; the general characters of waves, their frequency, the frequency gradient along the gut, their speed of transmission and integration of the waves.

General characters of the recorded waves.

Analysis of the kymograph tracings shows the following types of waves can be recorded from the gastric antrum, duodenal cap and jejunum.

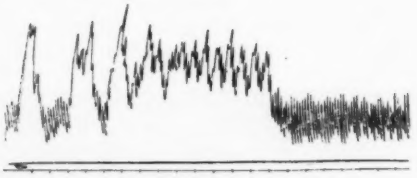

Fig. 4. Gastric tracing showing in order (i) large waves, (ii) tetany, (iii) quiescence (tonus rhythm). Note respiratory waves throughout, 14 per minute.

Respiratory waves. These can be seen in Fig. 4 and in most of the tracings reproduced. They result from variations in intra-abdominal pressure with respiratory movement, and are most apparent in gastric records.

Tonus rhythm. Tonus rhythm is represented by undulating shallow waves readily seen in the stomach (Fig. 4), and rarely seen in the duodenal or jejunal tracings. When tonus rhythm only is seen in the tracing, quiescence is said to be present.

Small waves (S). Small waves are simple monophasic waves usually of low amplitude. Fig. 5 shows them occurring in the stomach duodenum and jejunum intermixed with larger waves. Transition from gastric tonus rhythm → small gastric waves → large gastric waves is shown in Fig. 6[2], and this sequence commonly occurs after a period of quiescence. This tracing also shows S waves in the duodenum occurring in a rhythmic pattern which is only seldom seen.

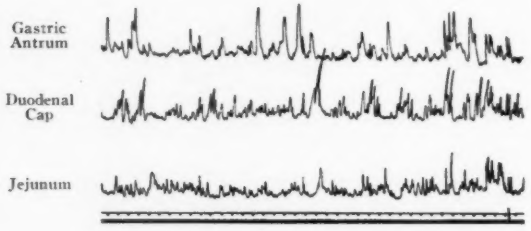

Fig. 5. Simultaneous record from gastric antrum duodenal cap and jejunum. Time=30 seconds.

The small waves have been designated S waves by Ingelfinger and Abbott (1940) and correspond to Type 1 waves of Templeton and Lawson (1931). They are probably segmenting or non-progressive contractions (Chapman *et al.*, 1949).

Their duration is 10-20 seconds.

[2]Although this tracing is from a patient with functional dyspepsia, and not one of the control series, it has been reproduced because of its clarity. There is no feature in this record which has not been seen in normal subjects.

Large waves (L). Large waves (L) are shown occurring in the stomach in Figs. 5, 6 and 7. They are monophasic of high amplitude, broad based and easily distinguished from short lived "spikes" due to artefacts *(vide infra).* Their duration is 20-60 seconds, tending to the shorter period. They are due to peristaltic contractions and represent the passage of a single wave over the balloon (Chapman *et al.,* 1949). They correspond with the "hunger contractions" of Carlson (1916), and with the Type II waves of Templeton and Lawson (1931) and Adler Atkinson and Ivy (1941).

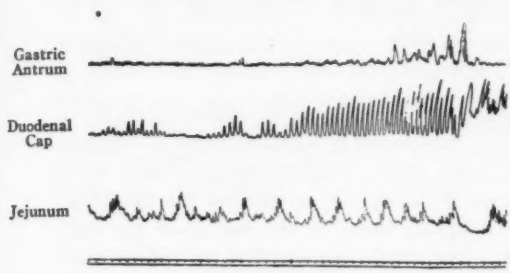

Fig. 6.

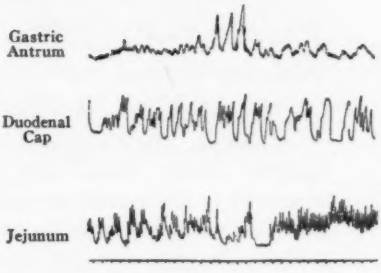

Fig. 7.

Fig. 6. Simultaneous record from gastric antrum duodenal cap and jejunum. Time= 30 seconds. S waves or Type I waves in duodenum. Tetany or Type III waves in jejunum. Note sustained tetany in duodenal and jejunal tracings towards end of record.

Fig. 7. Simultaneous record from gastric antrum, duodenal cap and jejunum. Time= 30 seconds. Note—(i) tetany or Type III waves in duodenal and jejunal tracings. The obviously slower rate of S waves compared with respiratory waves of the gastric tracing shows they are not due to respiration. (ii) Sustained tetany in jejunal tracing towards end of record.

Sometimes gastric L waves appear as a rhythmic pattern with a constant rate for short or long periods; at other times there occurs a non-rhythmic pattern composed of waves of a different type (large and small) intermixed, or periods of quiescence of variable length break up the sequence.

Code *et al.* (1952) state that Type II (large) waves are not seen in the small bowel, and they have not been found in these records in the typical monophasic form.

Tetany or spasm. Tetany or spasm, occurring in the stomach, is shown in Fig. 4. It is due to small (S) waves of high frequency (9-12 per minute) and low amplitude, superimposed on the balloon together with a sustained spasm which raises the baseline. It is uncommon in the stomach, where it endures for 1-6 minutes. The longer periods are usually at the termination of activity, being followed by a period of quiescence (in which tonus rhythm only is seen) lasting from a few minutes up to half an hour or more.

In the duodenum these complex waves are common, lasting for about 30 seconds as a rule (Fig. 7), but occasionally continuing for some minutes.

In the jejunum, tetany is the usual wave shape, lasting for 1-1½ minutes (Fig. 6). As in the other two sites, jejunal tetany may be sustained for several minutes (Fig. 7).

Tetany corresponds to Type III waves of Templeton and Lawson (1931).

Artefacts. Coughing, sneezing, talking, deep breathing, moving and urination will all produce waves. Their appearance on the record can be minimized by preliminary instructions to the subject, who is told to pinch the Miller-Abbott tube during the activities which produce them. They can usually be recognized by the sharp up and down stroke of short duration and their synchronous appearance in all three records.

Frequency of waves.

The frequency with which the waves recur varies in the different sites.

Stomach. In the stomach tonus rhythm occurs at a rate of about 1-3 undulations per minute. Small waves have similar rates. The frequency of large waves measured in the gastric antrum is usually about one or two per minute. Considerable variations may be seen at different times in the record of the same subject, the rate changing from one wave every two minutes to two or three waves per minute in the space of a few minutes.

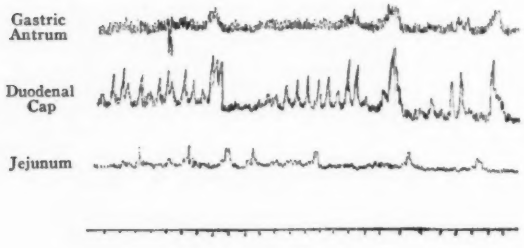

Fig. 8.

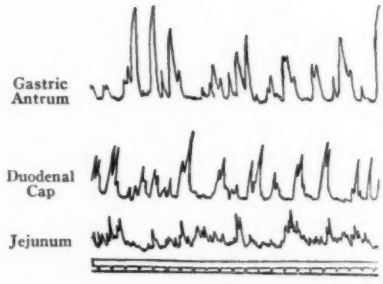

Fig. 9.

Fig. 8. Simultaneous record from gastric antrum, duodenal cap and jejunum. Time= 30 seconds.

Fig. 9. Simultaneous record from gastric antrum, duodenal cap and jejunum. Time= 30 seconds. Note harmonious waves passing from stomach→duodenum→jejunum.

Duodenum. In the duodenal cap, the frequency of waves is much more variable than in the antrum of the stomach. Rates are commonly seen of the order of one per minute, for tetany. Short waves usually occur at a rate of 3 per minute (Fig. 6 and Fig. 8) when rhythmic, but at higher rates (9–12 per minute) when they form one component of tetany waves (Fig. 6).

Jejunum. The rates of waves in the jejunum are similar to those in the duodenum. Slow rates for tetany waves are seen of one every 2-3 minutes

(Fig. 8), 6 waves in 10 minutes (Fig. 6) and 6 waves in 5 minutes (Fig. 7). As in the duodenum, S waves sometimes are inextricably mixed in with tetany waves, and the rate is often labile and irregular.

Frequency gradient.

It has been shown by Douglas (1948) that, in the dog, there is a fall in the frequency of rhythmic contractions at successive levels down the gut. The rate in the duodenum and jejunum he found to be the same, the decline not being appreciable until the ileum is reached.

In the present study no definite gradient in frequency is apparent between duodenum and upper jejunum. But the complex relationship between the waves in these two regions *(vide infra)* makes it very difficult to analyse the possibility of such an underlying gradient. Often the rates are clearly the same.

However, sections of the tracing chosen at random (Table 1) suggest that, from duodenum to jejunum, at times there is a gradient of rhythmicity, the wave rate being higher in the duodenum.

TABLE 1.

Frequency gradient. No. of waves observed in 10-minute periods.

Subject	Ki.		Ph.*	Fa.		Wo.	Wa.					Ro.
Duodenal cap	10	9	14	13	10	10	12	14	7	32	10	10
Jejunum	7	9	6	12	8	8	8	6	6	11	10	8

* Rate Ph. over 4 minutes.

Speed of wave transmission.

The time elapsing between waves which start in the gastric antrum and pass to the duodenal cap and thence to the jejunal site of the distal balloon has been measured. The limits between antrum and cap (a distance of 9 cm. from the centre of each balloon) were found to be 5-60 seconds, with an average of 15-20 seconds, giving an average rate of about 0·5 cm./sec. Exact measurements are not possible owing to the complexity of many wave forms.

Between the centre of the duodenal cap balloon and the centre of the distal balloon in the jejunum (12 cm.) waves were found to have intervals of 15-30 seconds, the average being approximately 20 seconds, a rate of about 0·5 cm./sec.

Integration of waves.

The analysis of results, so far, has been of single waves or groups of waves from various sites in the upper gastro-intestinal tract. When each record is considered as an integrated pattern of gut motilities, considerable difficulty arises in the interpretation, but certain broad underlying principles are apparent.

The two main principles appearing from this study are harmony and autonomy of gut activity between contiguous segments.

These principles appear to have escaped the emphasis they deserve in previous work on humans.

Patterson and Sandweiss (1942), in a study of the relationship between gastro-duodenal motility of normal subjects with hunger sensations, showed by a two-balloon technique (one in the antrum and one in the duodenum) that the following phases occurred:

1. Simultaneous gastric and duodenal motility.
2. Duodenal motility with a quiescent stomach.
3. Simultaneous gastric and duodenal quiescence.
4. Gastric motility with duodenal quiescence.

This work clearly showed that dissociation occurred at times between gastric and duodenal activity, although a paper of Hightower and Gambill (1953) is based on the wrong assumption that the pressure changes in the gastric antrum indicate the phasic pressure changes in the duodenal bulb.

In all our tracings, at some stage of the record, L waves can be seen in progress in an orderly manner from gastric antrum to duodenal cap and on to the jejunum (Fig. 9). Such an harmonious series may be limited to one wave, may continue for a minute or two, or they may continue for over an hour. These are clearly peristaltic waves subserving the onward passage of bowel contents. At times waves arise simultaneously in antrum and cap (Fig. 8). This would suggest stimuli for wave movement arriving via the vagus simultaneously at these sites while progressive waves are subserved by intrinsic nerves.

An interesting phenomenon of "dropped beats" is not infrequently seen, the gastric wave petering out before it reaches the duodenum, or, more commonly, the duodenal wave may fail to evoke a response in the jejunum, both observations being seen at a time of harmonious activity.

Autonomous activity is the second commonly observed principle. At times stomach, duodenum and jejunum are acting in a completely dissociated manner, and a large number of different combinations of simultaneous behaviour in the three regions can be found. The theoretical number of possibilities of the three main activities—quiescence, waves (L or S) and tetany—in the three regions examined is large. In practice a considerable number has been observed. Thus in Fig. 8 we find: stomach quiescent duodenum small waves 3/minute, jejunum 1/2-3 minutes. In the same tracing it can be seen that occasional waves are harmonious, starting in the stomach and passing to duodenum and on to jejunum. Fig. 6 shows dissociation of all three regions. Fig. 7 shows harmony predominating, but autonomy appearing in the jejunum as tetany. Many other combinations have been observed in other tracings.

These observations clearly indicate in the regions studied that, in the same subject, at different times, motility occurs in harmonious or autonomous ways.

SUMMARY.

Gastro-intestinal motility has been studied with a balloon-kymographic system of air-filled balloons in the gastric antrum, duodenal cap and upper jejunum using X-ray control. Records were taken from six normal, fasting subjects and one fasting subject with functional dyspepsia.

The general character of the waves, their frequency, duration, speed of transmission and integration have been analysed.

It has been shown that, in the contiguous gut segments studied, waves may occur in a harmonious and integrated pattern, or each segment may behave in an entirely autonomous way.

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THE PANCREATIC LESION IN ADULT MICE INFECTED WITH A STRAIN OF PLEURODYNIA VIRUS

2. VIRUS TITRES IN THE PANCREAS AND THEIR CORRELATION WITH BLOOD VIRUS LEVELS AND MORPHOLOGICAL CHANGES[1]

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(Accepted for publication 7th December, 1953.)

Pathological changes in the pancreas of the adult mouse and the time of occurrence of the maximum blood virus level following intraperitoneal injection of the PL. 5 virus have been described (Dineen and Barter, 1953). This work and further studies on the appearance of infected tissue with the electron microscope (Robertson, 1954), indicated that the titration of pancreas harvested at intervals following injection of the virus would assist the interpretation of the complex morphology of the disease process.

The present investigation has yielded data from which the titre of virus has been extracted by the probit iterative approximation to the maximum likelihood estimate of the LD_{50} (Finney, 1952). These levels have been correlated with the development of the pancreatic lesion and the temporal occurrence of the maximum virus concentration in the blood stream.

Materials and Methods.

Preparation of virus suspensions.

From infant mouse muscle. Moribund animals from the 19th passage of the PL. 5 virus in infant mice were decapitated, and both fore and hind limbs harvested and stored at −30° C.

The limbs were homogenized in a mortar with alundum and a 20 p.c. suspension was prepared in chilled physiological saline. The crude suspension was spun in a "Spinco" ultracentrifuge at 15,000 r.p.m. for 10 minutes and the supernatant harvested. After demonstration of bacteriological sterility, the preparation was stored at −30° C.

From adult mouse pancreas. Pooled pancreases from infected adult animals were weighed and homogenized in a mortar and a 10 p.c. suspension was prepared in chilled physiological saline. The crude suspensions were centrifuged at 10° C. for 20 minutes at 3,000 r.p.m. and the supernatant fluids harvested. After bacteriological sterility had been demonstrated the suspensions were titrated.

Adult mouse inoculation.

Fifty-five 8-week old male mice were randomized and injected intraperitoneally with 0·2 ml. of the 19th passage infected infant mouse muscle suspension. The pancreases were harvested at 11 intervals which progressed geometrically from 6 hours to 15 days after injection. Five animals were sacrificed at each interval and the pooled pancreatic tissues were titrated in day-old infant litters.

[1]This work was carried out with support from the Medical Research Committee of the University of Adelaide and by the John Mortlock Medical Bequest.

Titration of the pancreas suspension in infant litters.

Serial tenfold dilutions of the 6-, 9- and 14-hour specimens were prepared in physiological saline from the 10^{-1} to the 10^{-7} dilution. Similar dilutions up to and including the 10^{-8} dilution were prepared from the 21-hour, 31-hour, 2-day, 3-day, 4½-day and the 7-day specimens. Serial double dilutions of the 10-day and 15-day specimens were prepared from the $10^{-1.6990}$ to the $10^{-3.3031}$ dilution. Each dilution was injected into two day-old litters by the interscapular route. Infant animals from groups of 14 litters were randomly redistributed among the does. Seven infant mice were included in a litter and each animal was injected with 0·02 ml. of the appropriate dilution. Infants dying within 24 hours of inoculation were discarded and the litters were observed twice daily for 14 days and the number of deaths recorded.

RESULTS.

The results are given in Table 1.

***TABLE 1.**

Virus titres in the pancreas.

Period between inoculation and harvesting of pancreas	Log_{10} dilution x	No. animals in batch n	No. animals killed r	Proportion killed p	
P 1 (6 hours)	—7	14	0	0·00	$y=7\cdot7935+0\cdot7255x$
	—6	14	1	0·07	$m=-3\cdot8504$
	—5	13	2	0·15	$V(m)=0\cdot056904$
	—4	14	7	0·50	$1/V(b)=67\cdot422$
	—3	14	12	0·86	$\chi_3^2=2\cdot919$
	—2	14	11	0·79	
	—1	14	14	1·00	
P 2 (9 hours)	—7	14	0	0·00	$y=10\cdot4866+1\cdot1473x$
	—6	13	1	0·08	$m=-4\cdot7822$
	—5	14	5	0·36	$V(m)=0\cdot034640$
	—4	14	13	0·93	$1/V(b)=20\cdot397$
	—3	14	13	0·93	$\chi_1^2=1\cdot448$
	—2	14	14	1·00	
	—1	14	14	1·00	
P 3 (14 hours)	—7	14	0	0·00	$y=9\cdot0531+0\cdot8544x$
	—6	14	1	0·07	$m=-4\cdot7438$
	—5	14	9	0·64	$V(m)=0\cdot046735$
	—4	14	10	0·71	$1/V(b)=45\cdot778$
	—3	14	12	0·86	$\chi_3^2=4\cdot7222$
	—2	14	14	1·00	
	—1	14	14	1·00	
P 4 (21 hours)	—8	13	2	0·15	$y=8\cdot7945+0\cdot6349x$
	—7	14	3	0·21	$m=-5\cdot9765$
	—6	14	5	0·36	$V(m)=0\cdot072625$
	—5	14	12	0·86	$1/V(b)=72\cdot594$
	—4	14	13	0·93	$\chi_3^2=2\cdot757$
	—3	14	13	0·93	
	—2	14	14	1·00	
P 5 (31 hours)	—8	14	4	0·29	$y=10\cdot2516+0\cdot6842x$
	—7	14	11	0·79	$m=-7\cdot6755$
	—6	14	14	1·00	$V(m)=0\cdot13047$
	—5	14	12	0·86	$1/V(b)=32\cdot172$
	—4	14	14	1·00	$\chi_3^2=7\cdot3185$
	—3	14	14	1·00	
	—2	14	14	1·00	

TABLE 1 (continued).

Period between inoculation and harvesting of pancreas	Log_{10} dilution x	No. animals in batch n	No. animals killed r	Proportion killed p	
P 6 (48 hours)	—8	14	3	0·21	$y=7·4582+0·3711x$
	—7	14	5	0·36	$m=-6·6241$
	—6	14	11	0·79	$V(m)=0·20659$
	—5	14	13	0·93	$1/V(b)=153·64$
	—4	14	10	0·71	$\chi_4^2=8·226$
	—3	14	11	0·79	
	—2	14	14	1·00	
P 7 (72 hours)	—8	13	1	0·08	$y=9·3760+0·7184x$
	—7	14	4	0·29	$m=-6·0913$
	—6	14	6	0·43	$V(m)=0·06172$
	—5	13	12	0·92	$1/V(b)=60·177$
	—4	14	12	0·86	$\chi_3^2=2·7964$
	—3	14	14	1·00	
	—2	14	14	1·00	
P 8 (4½ days)	—8	13	0	0·00	$y=9·0423+0·7614x$
	—7	14	2	0·14	$m=-5·3090$
	—6	14	5	0·36	$V(m)=0·05228$
	—5	14	7	0·50	$1/V(b)=63·70$
	—4	14	11	0·79	$\chi_3^2=0·848$
	—3	14	14	1·00	
	—2	13	13	1·00	
P 9 (7 days)	—8	13	0	0·00	$y=8·1564+1·1710x$
	—7	13	0	0·00	$m=-2·6955$
	—6	14	0	0·00	$V(m)=0·03873$
	—5	12	0	0·00	$1/V(b)=12·109$
	—4	14	1	0·07	$\chi_1^2=0·009$
	—3	14	5	0·36	
	—2	14	11	0·79	
P 10 (10 days)	—3·8081	14	0	0·00	$\log_{10} LD_{50}<-2$
	—3·5051	13	0	0·00	
	—2·9031	14	1	0·07	
	—2·6021	14	2	0·14	
	—2·3010	14	0	0·00	
	—2·0000	14	1	0·07	
	—1·6990	14	2	0·14	
P 11 (15 days)	—3·8081	14	0	0·00	$\log_{10} LD_{50}<-2$
	—3·5051	13	0	0·00	
	—2·9031	14	0	0·00	
	—2·6021	14	0	0·00	
	—2·3010	13	1	0·08	
	—2·0000	14	3	0·21	
	—1·6990	14	4	0·29	

y=the expected probit.

m=the $\log_{10}$ LD_{50}, i.e., x when $y=5$.

$V(\ )$=variance of.

b=estimate of the regression coefficient.

χ_r^2="goodness of fit" on r degrees of freedom.

Where n is <14 the animals which completed the batch died within 24 hours and therefore death was not due to infection.

* The number of decimals shown, 4, was for calculation only.

The first step in the analysis of the data was to transform the proportion killed to probits and plot these against the logarithm of the corresponding dilution for each pancreas specimen titrated. As these graphs, with the possible exception of that for the 48-hour specimen, appeared reasonably linear, probit analyses were carried out for each specimen. Analyses were not carried out for P 10 and P 11 as insufficient data were available for these specimens. The graph for the 48-hour specimen was unusual in that a decrease in the proportion killed occurred with increasing concentration at dilutions 10^{-4} and 10^{-3}. The results of the probit analysis of each titration, including the regression equation, the logarithm of the LD_{50}, the variance of the LD_{50}, the Chi-square "goodness of fit" on r degrees of freedom and the reciprocal of the variance of b, the regression coefficient which was used in subsequent computations, are given in the sixth column of Table 1.

Because the expected number killed at several dilutions in each titration was small (less than 1), the χ^2 to test goodness of fit had to be calculated from the expectations obtained by pooling some of the dilutions instead of by the simpler method which the probit analysis supplies when all expectations are reasonably large. The χ^2 therefore had varying degrees of freedom, as shown by their suffixes; those estimated on one degree of freedom were probably not worth calculating. Only P 5 had a significant value attaining the 5 p.c. level although the χ^2 of P 6 is close to significance at this level.

The differences of virus titres between specimens was assumed to be due only to differences in the "concentration" of virus in the specimens. Consequently the regression lines, of which the analysis gave estimates, should all have the same slope. In other analyses with more extensive data, this assumption would have been tested by carrying out an analysis in which individual a's and a common b were estimated, giving a total χ^2 for goodness of fit, i.e., χ_c^2; then if χ_s^2 was the total χ^2 for the probit analysis carried out above where individual a's and individual b's were estimated,

$$\chi_\beta^2 = \chi_c^2 - \chi_s^2$$

is the χ^2 to test the hypothesis of a common slope. Because a few degrees of freedom were available for some of the titrations, an approximate test was performed which was in effect a weighted analysis of variance.

If each b was normally distributed about a common value β with variance σ_i^2 then

$$\sum_1^k \frac{(b_i - \bar{b})}{\sigma_i^2} \text{ is a } \chi_{k-1}^2 \text{ where } w_i = \frac{1/\sigma_i^2}{\Sigma\, 1/\sigma_i^2} \text{ and } \bar{b} = \Sigma w_i\, b_i$$

The w_i have been chosen so that $\bar{b}$ was an unbiased estimate of β, i.e., $E(\bar{b}) = \beta$ and subject to this the variance of $\bar{b}$ is a minimum. The use of these

weights which are proportional to $1/\sigma_i^2$ reflected the fact that more importance was attached to those b's which are more precise, that is with smaller variances. In the absence of σ_i^2 which were not known, their best estimates V (bi), whose reciprocals appear in Table 1, were substituted. Table 2 shows the calculations.

TABLE 2.

Weighted analysis of variance.

Specimen	b	$1/V(b)$	$\frac{(b-\bar{b}_1)^2}{V(b)}$	$\frac{(b-\bar{b}_2)^2}{V(b)}$ omitting P 6
P 1	0·723	67·422	0·3792	0·1025
P 2	1·147	20·397	5·0789	3·0233
P 3	0·854	45·778	1·9426	0·3875
P 4	0·635	72·594	0·0123	1·1709
P 5	0·684	32·172	0·0417	0·1957
P 6	0·371	153·64	11·7886	—
P 7	0·718	60·177	0·2949	0·1165
P 8	0·761	63·700	0·8134	0·0001
P 9	1·171	12·109	3·3122	2·0256
			χ_8^2=23·6638	χ_7^2=7·0221

$\bar{b}_1$=342·202/527·989 =0·648 $\bar{b}_2$=285·202/374·349 =0·762

The significance of the χ^2 on 8 degrees of freedom was due almost entirely to the low value of $b6$. When this b was omitted, the new χ^2, calculated on 7 degrees of freedom, was not significant. These calculations agree with observations that are evident from Fig. 1, in which the regression coefficients have been graphed against the $\log_{10}$ of the time.

The hypothesis of a common slope was not rejected as the significance of the b's was attributed to P 6 being a poor estimation. This assumption was considered legitimate on the basis that P 6 was an unusually large deviation which may have been due to the mice at dilutions 10^{-5} being particularly susceptible.[2]

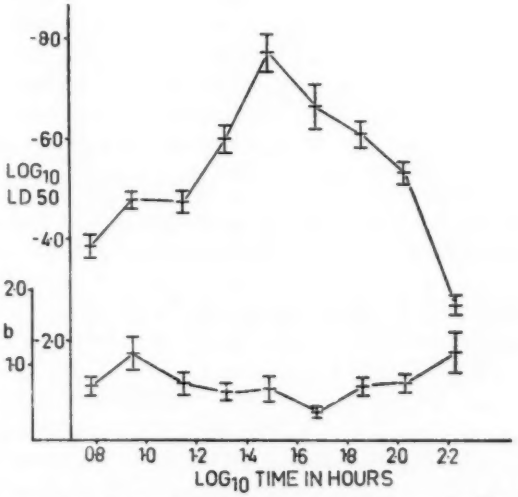

Fig. 1. Graphs of the LD_{50}'s and the corresponding regression coefficients, b, with 1 σ bands against the $\log_{10}$ of the time.

[2]In further work the hypothesis of a common slope will be a point to be watched and checked.

The logarithm of the LD_{50} of each specimen has been plotted against the $\log_{10}$ of the time in hours which elapsed between inoculation of the adult mice and harvesting the pancreas (see Fig. 1).

Table 3 shows the log LD_{50}'s m_i and their estimates of variance. An approximate test of differences between two consecutive log LD_{50}'s m_i and m_{i+1} was carried out by forming

$$u = \frac{m_{i+1} - m_i}{\sqrt{V(m_{i+1}) + V(m_i)}}$$

and assuming this to be normally distributed. The calculations are given in Table 3.

TABLE 3.

Significance of differences between consecutive LD_{50}s.

Specimen	Log LD_{50} m	$V(m)$	$\frac{m_{i+1}-m_i}{\sqrt{V(m_{i+1})+(Vm_i)}}$
P 1	—3·85	0·05690	3·07†
P 2	—4·78	0·03464	0·14 N.S.
P 3	—4·74	0·04674	3·59‡
P 4	—5·98	0·07263	3·77‡
P 5	—7·68	0·13047	1·83 N.S.
P 6	—6·62	0·20659	1·02 N.S.
P 7	—6·09	0·06172	2·31*
P 8	—5·31	0·05228	8·65‡
P 9	—2·70	0·03873	

N.S.=no significance.

*=significance at the 5 p.c. level.

†=significance at the 1 p.c. level.

‡=significance at the 0·1 p.c. level.

The results obtained in Table 3 confirmed the interpretation of the graph of the log LD_{50}'s of the specimens against time in that the 0·1 p.c. level of significance has been attained between P 3 and P 4, P 4 and P 5, and P 8 and P 9, the 1 p.c. level between P 1 and P 2, and the 5 p.c. level has been attained between P 7 and P 8, but no significant difference in virus titres has been demonstrated between P 2 and P 3, P 5 and P 6, and P 6 and P 7.

DISCUSSION.

The method of probit analysis for estimating the LD_{50}'s of the specimens was preferred because the virus concentrations in the pancreas could not be predicted before titration. The methods of Behrens (1929) and Kärber (1931) require this knowledge, as a series of dilutions of virus covering the range of kills from 0–100 p.c. is necessary. The Behrens method is further unsuited to the virus titrations because in it equal numbers of animals in the batch are

necessary. In this laboratory the method of virus titration in the infant mouse was associated with a small mortality rate due to the technique of injection or failure of the does to suckle all the infants. In the present series of titrations a mortality rate of 1·3 p.c. was attributed to these causes. Furthermore, as the estimation of the variances of the LD_{50}'s was necessary for the computation of a statistic to demonstrate a difference between virus titres of consecutive specimens the method of Reed and Muench (1938) is also unsuited to the present problem.

Although the Reed and Muench method does not supply the variances of the LD_{50}'s, when this calculation was applied to the titration data results were obtained which very closely approximated the LD_{50}'s estimated by probit analysis. The greatest difference between corresponding LD_{50}'s computed by the two methods occurred in the titration at 48 hours and was 0·24 $\log_{10}$.

During the course of the PL. 5 virus infection in the adult mouse the titre of virus in the pancreas increased from an LD_{50} of $10^{-3.85}$ at 6 hours to $10^{-7.68}$ at 31 hours. The decrease of the virus concentration from the 31-hour specimen to the 48-hour specimen was not significant according to the statistic u, but the estimation of this value detected a significant difference between the P 5 (31 hours) and P 7 (72 hours) specimens at the 0·01 p.c. level (u=3·63). Consequently the maximum titre of virus in the pancreas occurred between 21 and 72 hours after intraperitoneal inoculation, and the results of the present experiment suggested that this level occurred at about 31 hours.

Since the experimental conditions and the animal colony were the same, it was perhaps permissible to relate the present findings to those previously reported by Dineen and Barter (1953), who showed that the maximum concentration of virus was detected in the blood stream between 36 and 72 hours after intraperitoneal injection, and considered that the pancreas was the major source of virus for the blood stream. The present investigation supported this hypothesis because the maximum concentration of virus in the blood stream occurred at about the same time as the maximum level in the pancreas. In addition, when the LD_{50} was low (less than 10^{-2}) in the pancreas at 10 days, the methods used in the earlier experiment could not detect virus in the blood stream.

A continued decrease of the virus titres was demonstrated by LD_{50}'s of $10^{-5.31}$ in the 4½-day specimen and $10^{-2.70}$ in the 7-day specimen, and thereafter a gradual "tailing off" of the virus concentration in the pancreas was indicated by the low level of virus in the specimens harvested 10 and 15 days after inoculation. The LD_{50}'s of these specimens were less than 10^{-2}.

The earliest lesion observed in the pancreas was characterized by a scanty inflammatory reaction beginning about 12 hours (Robertson, 1954) after intraperitoneal injection of the virus. The LD_{50} of virus increased from $10^{-3.85}$ at 6 hours in P 1 to $10^{-4.78}$ at 9 hours, and therefore a significant amount of virus multiplication had occurred before a reaction of the host was manifested. During

the following 17 hours the LD_{50} rose to $10^{-7.68}$ in the 31-hour pancreas, and this virus multiplication may be correlated with an increase of the inflammatory response in the host which reached a peak about 36 to 48 hours after virus injection.

In the earlier communication (Dineen and Barter, 1953) the occurrence of "abnormal structures of roughly spherical appearance" in the basophilic portions of the pancreatic cells at 48 hours was described, and their origin was attributed to either degenerating cytoplasm or virus inclusion bodies. A much more detailed morphological analysis has since been made with the electron microscope (Robertson, 1954), which also showed that the changes in the pancreatic acinar cells reached their greatest complexity at 36 to 48 hours, and further, that the reaction was quite different in character from that resulting solely from post-mortem changes in these cells. The virus titre in the pancreas at this stage was high (the LD_{50} at 48 hours was $10^{-6.62}$). Consequently, it seems reasonable to relate these morphological changes to the results of virus action.

From the 4th to the 12th days after inoculation of the virus into the adult mouse fibroblastic proliferation was observed, and during this period a considerable fall in the virus titre in the pancreas has now been demonstrated.

The present investigation has shown a relationship between the virus concentration in the pancreas, the virus concentration in the blood stream, and the features of the pathological changes in the pancreas.

SUMMARY.

The LD_{50}'s of infected pancreas have been estimated by probit analysis from specimens harvested from 6 hours to 15 days after intraperitoneal injection of the PL 5 virus into 8-week old male mice.

The LD_{50} at 6 hours was $10^{-3.85}$, which rose to $10^{-7.68}$ at 31 hours; thereafter the level fell to less than 10^{-2} at 10 and 15 days.

The maximum level of the virus in the pancreas coincided with the occurrence of the maximum level in the bloodstream, which was determined in an earlier experiment (Dineen and Barter, 1953). In addition, when the LD_{50} was low (less than 10^{-2}) in the pancreas at 10 days, the methods used in the earlier work could not detect virus in the bloodstream.

Considerable multiplication of the virus in the pancreas had occurred before the inflammatory response which was evident 12 hours after injection.

The rise in titre of the virus in the pancreas has been correlated with a series of complex changes in the acinar cells which reached a peak about 36 to 48 hours after virus injection. The virus titre was still high at $4\frac{1}{2}$ days ($10^{-5.31}$), at which time the acinar cells were almost completely necrotic and replaced by mononuclears.

From the 4th to the 12th days after inoculation of the virus into the adult mouse fibroblastic proliferation was observed, and during this period a considerable fall in the virus titre in the pancreas has now been demonstrated (the LD_{50} at $4\frac{1}{2}$ days was $10^{-5.31}$, and at 10 days less than 10^{-2}). The virus titre in the pancreas was highest between 21 and 72 hours with a probable peak value of $10^{-7.68}$ at 31 hours, shortly before the morphological changes in the acinar cells reach their greatest complexity.

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CARDIAC OUTPUT IN NORMAL UNANAESTHETIZED AND ANAESTHETIZED RABBITS[1]

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Over the last few years unanaesthetized rabbits have been extensively used in this laboratory in investigating cardiac output changes by the Fick method under a variety of conditions. The circulatory effects of anoxia (Courtice and Korner, 1952; Korner, 1954), noradrenaline (Korner, 1953) and haemorrhagic anaemia (Darian Smith and Simmonds, 1954; Darian Smith, 1954) have been investigated. The results with noradrenaline and anaemia are in general agreement with other work in animals and man. The response to anoxia was somewhat different from the results reported in the literature and this is fully discussed elsewhere. The present paper summarizes our results in normal unanaesthetized animals.

METHODS.

Animals. Rabbits of mixed breeds varying in weight from 1·2 to 3 kg. were used. Most of the animals weighed 1·5 to 2·5 kg.

Cardiac catheterization. The operation was done with a combination of very light general anaesthesia and local subcutaneous infiltration of 1 p.c. procaine hydrochloride. The general anaesthesia was just adequate to make the animal lie quietly on the operating table. Veterinary nembutal (Abbott 60 mg./ml.) was used intravenously for one group of rabbits. Dosage was judged by the response of the animal and was usually about 0·4 ml./kg. body weight. Sodium pentothal (20 mg./ml.) was used for a second series, the dose being 0·5 to 0·7 ml./kg. In normal animals nembutal was more regular in action and more convenient to use. However, its effects lasted longer and delayed the experiment. Pentothal was satisfactory because of its short duration of action, and because it caused no deterioration in the condition of grossly abnormal animals (e.g., severely anaemic animals). The right common carotid artery and right external jugular vein were exposed through a midline incision. A polythene catheter (1·2 mm. external bore) filled with heparinized saline was inserted into the external jugular vein just below the bifurcation and passed 6 cm. The position of the tip was determined by the ease of withdrawing a sample of blood. Only when the catheter was in the atrium could blood be drawn back readily without partial or complete obstruction. The position of the catheter tip was always checked post-morten. Only twice in 120 catheterizations was a sample of 2 ml. of blood obtained through a catheter not in the atrium. Occasionally

[1]This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

the catheter passed into the right ventricle. The right common carotid artery was catheterized with Transflex tubing of similar bore. Transflex was found to be far more satisfactory in the artery than polythene, because of the less frequent clotting at the catheter tip. A glass L-shaped tracheotomy tube was tied into the trachea and the incision sutured in layers. The average duration of the operation was about 20-30 minutes, and no experiments were begun until at least 3 hours after the operation, by which time the animal was very alert and active.

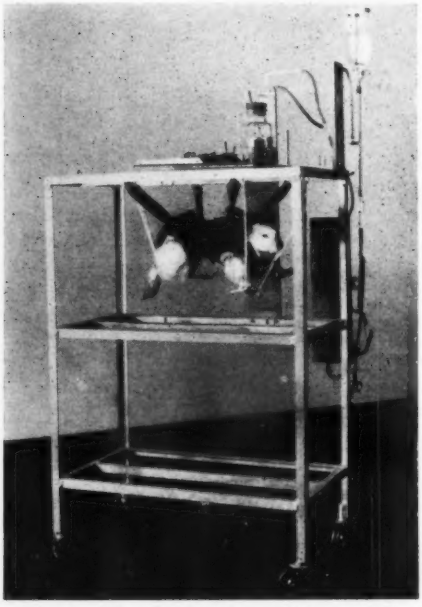

Fig. 1. Unanaesthetized rabbit suspended from the metal trolley. Limbs are quite loosely restricted.

Fig. 2. Recumbent animal on detachable platform. The respiratory valve and the catheters are clearly seen.

To allow the unanaesthetized rabbits moderate freedom during the experiment, the trolley seen in Figs. 1 and 2 was constructed. While still anaesthetized the animal was placed into a leather bag so made that the body weight was supported mainly by the fore and hind limbs. The bag had several large holes cut into its side to permit adequate ventilation. The animal recovered from the anaesthetic resting on the detachable platform seen in Fig. 2. When about to start the experiment, this was removed and the animal suspended (Fig. 1). In a series of normal animals and in abnormal states (e.g., severe anaemias) the whole experiment was done with the animal recumbent.

Air collection. The air valves used were of the simple rubber flap type, carefully tested for leaks, with a dead space of about 2 mls. (Fig. 2). Gas samples were collected for a period of 2-3 minutes in a rubber bag of 4-litre capacity. A sample of expired air was always stored in the bag for 24 hours before each experiment to saturate the rubber with CO_2. Gas volumes were measured immediately after each collection with a standard gas meter, samples

collected in sampling tubes and the air analyses done with a Haldane air gas analysis apparatus.

Blood samples. Right atrial and arterial blood samples were withdrawn immediately after air collection. This procedure disturbed the rabbits much less than the simultaneous collection of the two sets of samples. In ten experiments simultaneous blood and air collections were carried out and compared with the results of blood collected immediately after the air collection. There was no significant difference in the estimates of cardiac output obtained in these two ways. The blood was collected in airtight paraffined syringes and the samples stored in glass tubes under paraffin in crushed ice. Dry heparin was used as the anticoagulant and analyses for O_2 content, O_2 capacity and CO_2 content were always done within 3 hours of collection, using the modified Haldane Blood Gas Apparatus (Courtice and Douglas, 1947). The adequacy of this method of storage was tested for both O_2 content and CO_2 content and found to be quite satisfactory. Immediate storage in ice prevented glycolysis over 4 hours, as was previously reported by Lovatt Evans (1922).

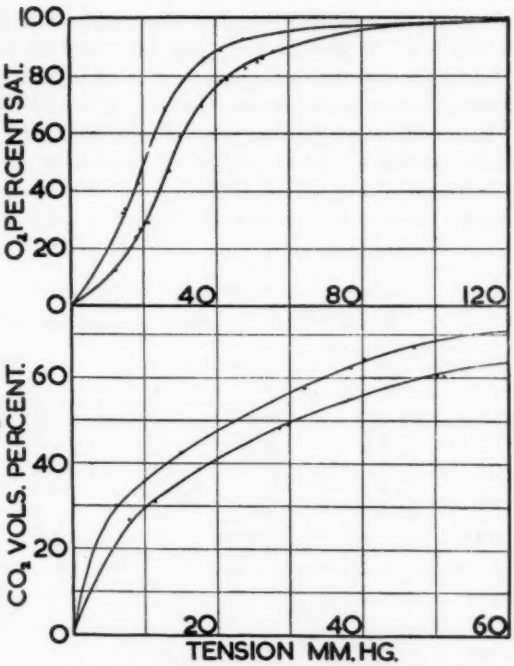

Fig. 3. Oxygen and carbon dioxide dissociation curves for rabbit blood. CO_2 tension for the upper oxygen dissociation curve was 15 mm. Hg. and for the lower 40 mm. Hg. Fully oxygenated blood was used for the lower CO_2 dissociation curve and fully reduced blood for the upper curve.

O_2 and CO_2 dissociation curves. The oxygen dissociation curve is required in the calculation of the correction due to dissolved oxygen in the process of blood gas analysis, and for this reason a curve was constructed for rabbits' blood (Fig. 3). Dry heparin was used as the anticoagulant and 0·1 p.c. previously heated sodium fluoride was added to the samples. Standard oxyhaemoglobin dissociation curves were constructed at CO_2 tensions of 40 mm. Hg. and 15 mm. Hg. (Douglas and Priestley, 1948). CO_2 dissociation curves were also constructed with aerated and reduced blood. These curves for rabbits' blood are published because of their inaccessibility in the English literature.

Pressure measurements. Mean carotid blood pressure was measured directly with a mercury manometer. Atrial pressures were measured using a modification of the method of Burch and Winsor (1943).

Stability of preparation. Most of the experiments of this type done in this laboratory have been terminated within 10 hours of giving the initial anaesthetic, and within these limits the preparation appears quite stable.

RESULTS.

The errors in the determination of oxygen consumption, A-V oxygen difference and cardiac output.

Oxygen consumption. An experiment was designed in which the oxygen consumption was measured at different time intervals before and after determination of a cardiac output. The results and analysis of variance are shown in Table 1. It may be seen that the standard error of a single determination of oxygen consumption, measured from 15 minutes before the cardiac output determination to as long as 4 hours after it, is 3 ml. (i.e., 11 p.c. of the mean value of 26 ml.). Of interest from the point of view of attainment of a "steady state" during a cardiac output determination are the results of duplicate determinations of oxygen consumption 15 minutes before and 15 minutes after a cardiac output determination (Group A and B of Table 1). The standard error of a single determination is 0·7 ml. (i.e., 3 p.c. of the mean value).

TABLE 1.

Reproducibility of oxygen consumption (ml./min.) determination.

Group A		Group B		Group C		Group D	
T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
29·0	29·5	19·0	18·0	38·9	48·2	31·7	40·5
25·6	24·4	27·6	27·2	30·7	31·5	30·7	31·5
19·3	20·1	23·0	24·1	20·8	22·6	27·6	19·1
23·0	22·9	18·3	17·6	19·6	19·0	19·0	17·9
21·0	20·7	22·0	23·3	33·6	36·0	32·6	40·8
23·3	22·0	24·5	26·3	18·2	21·8	22·9	22·6

Analysis of variance of Groups A, B, C, D.

Source of variation	Sum of squares	D/F	Mean square
Between groups	336	3	112
Between times	14	1	14
Times x groups	17	3	6
Between animals	2127	23	92
Error	153	17	9
Analysis of variance of Groups A and B.			
Between animals	260	11	23·5
Error	6	12	0·5

Group A—Duplicate determination 15 min. before cardiac output determination.
Group B—Duplicate determination 15 min. after cardiac output determination.
Group C—Duplicate determination 1 hr. after cardiac output determination.
Group D—Duplicate determination 4 hr. after cardiac output determination.
T_1—Time of cardiac output estimation.
T_2—Second time interval.

A-V oxygen difference. To gain an idea of the physiological fluctuations in A-V oxygen difference, an experiment was designed in which cardiac outputs were determined either 1, 4 or 5 hours after doing a control cardiac output. The

results are shown in Table 2. The A-V oxygen difference was affected similarly at the three time intervals. The standard error of a single determination was 0·91 volumes p.c. (14 p.c. of the mean value). The causes for the fluctuations are considered below.

TABLE 2.

Reproducibility of A-V O_2 difference and cardiac output at different time intervals.

A-V O_2 difference (vols. p.c.).

Group A		Group B		Group C	
T_1	T_2	T_1	T_2	T_1	T_2
7·3	6·9	7·5	8·4	6·6	6·7
7·9	8·2	10·6	9·3	4·5	4·2
4·0	5·2	4·0	4·6	4·8	3·4

Analysis of variance.

Source of variation	Sum of squares	D/F	Mean square
Between groups	17·3	2	8·7
Between times	0·2	1	0·2
Times×groups	0·2	2	0·1
Between animals	52·8	8	6·6
Error	3·3	4	0·83

Cardiac output (ml./min.).

Group A		Group B		Group C	
T_1	T_2	T_1	T_2	T_1	T_2
529	698	422	482	500	611
459	423	307	338	210	238
528	456	499	568	578	559

Analysis of variance.

Source of variation	Sum of squares	D/F	Mean square
Between groups	21752	2	10876
Between times	6460	1	6460
Times×groups	827		413
Between animals	222736	8	27842
Error	28088	4	7028

Group A—Duplicate estimation 1 hr. apart.
Group B—Duplicate estimation 4 hr. apart.
Group C—Duplicate estimation 5 hr. apart.

T_1—Time of first determination.
T_2—Time of second determination.

Cardiac output. A similar design as used in assessing the error of a determination of A-V oxygen difference was used here and the results are shown in Table 2. The mean cardiac output was 467 ml./min. and the standard error of a single determination was 85 ml. in this series (18 p.c. of the mean). This agrees with the results of Donald, Bishop, Cumming and Wade (1953) in man.

Physiological factors affecting oxygen consumption, A-V oxygen difference and cardiac output.

The results quoted in this section are based on 55 cardiac output determinations carried out on suspended unanaesthetized rabbits. The mean results of oxygen consumption, A-V oxygen difference, cardiac output, arterial and venous oxygen saturation and CO_2 content, as well as blood pressure and heart rates and respiratory data, are summarized in Table 3 together with the 95 p.c. fiducial probability limits. In connexion with the statistical procedures used in determining the effects of various physiological factors on oxygen consumption, A-V oxygen difference and cardiac output, it is obvious that the estimated cardiac output is the ratio O_2 consumption/A-V O_2 difference. As will be seen, these two quantities are not independent. Factors affecting the separate components were analysed before considering the cardiac output.

TABLE 3.

Cardiorespiratory data in suspended unanaesthetized rabbits.

	Weight kg.	O_2 consumption ml./min.	Ventilation ml./min.	R.Q.	Arterial saturation p.c.	Mixed venous saturation p.c.	Arterial CO_2 content vols. p.c.*	A-V oxygen difference vols. p.c.	Cardiac output ml./min.	Mean arterial pressure mm. Hg.	Heart rate/ min.	Stroke volume min.
Mean	1·9	24·9	1060	0·82	97	61	44	6·1	415	137	290	1·4
Standard deviation	0·41	7·1	500	0·094	1·36	7·96	1·7	1·52	110	13	60	—
95 p.c. fiducial limits	1·1– 2·7	10– 40	—	0·64– 0·98	94·3– 99·8	45– 77	40– 48	3·0– 9·1	195– 635	111– 163	170– 410	—

* Estimation done in 27 cardiac output determinations.

All other values based on 55 cardiac output determinations.

Oxygen consumption. The effects of body weight and ambient room temperature on oxygen consumption are given in the first regression equation of Table 4. The equation indicates that oxygen consumption was directly related to body weight and rose about 13 ml. for a rise in body weight of 1 kg. The oxygen consumption was inversely related to the ambient room temperature within the temperature range of the experiment (26-15° C.) and rose about 3 ml./kg./min. for each 10° C. fall in room temperature. These inter-relationships are represented graphically in the nomogram of Fig. 4.

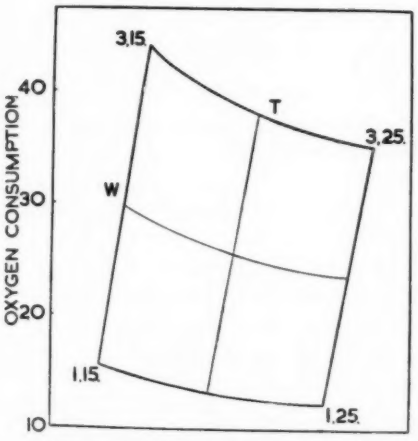

Fig. 4. Nomogram representing the relation between oxygen consumption in ml./min., S.T.P., body weight (W) in kg., and room temperature (T) in degrees C. The co-ordinates of each point are expressed in the above order (W, T). Log units in regression equation have been converted.

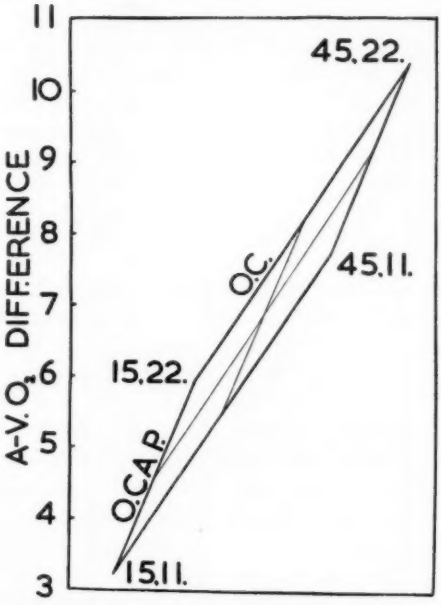

Fig. 5. Nomogram representing the relation between A-V oxygen difference in ml./100 ml. blood and oxygen consumption (O.C.) in ml./min., S.T.P., and oxygen capacity (O.CAP.) in ml./100 ml. blood. The co-ordinates of each point are expressed in the above order (O.C., O.CAP.). The weight is the mean value (1·9 kg.) for the series.

A-V oxygen difference. The second regression equation of Table 4 shows that the A-V oxygen difference was directly related to the oxygen consumption/min. and to the oxygen capacity of the blood (haemoglobin). It rose by 0·15 volumes p.c. for an increase in the rate of oxygen consumption of 1 ml./min. and by 0·24 volumes p.c. for a change in O_2 capacity of 1 volume p.c. (experimental range of O_2 capacity was 11·5 volumes p.c. to 22·5 volumes p.c.). This relationship may be read off directly from the nomogram in Fig. 5.

TABLE 4.

Relation of various factors to O_2 consumption, A-V O_2 difference and cardiac output in suspended, unanaesthetized rabbits.

	No. of results	Regression equation	Significance of regression coefficients b_1	b_2	b_3	Sum of squares of dependent variate	Sum of squares removed by partial regression coefficients b_1	b_2	b_3	Residual sum of squares of dependent variate
Oxygen consumption	55	log OC=1·386+0·96 log (W—0·270) —0·47 (log (T—1·302)	p 0·001	p 0·001	p	0·877	0·465	0·119	—	0·293
A-V O_2 difference	54	AVO=6·1+0·15 (OC—25·2)+0·24 (OCAP—16·4)—0·92 (W—1·9)	p 0·001	p= 0·01	p= 0·2	139·8	69·8	16·6	—11·9	65·3
Cardiac output	55	log CO=2·618+0·691 log (W—0·276) —0·57 log (OCAP—1·213) —0·49 log (T—1·302)	p 0·001	p= 0·07	p= 0·02	0·9675	0·1991	0·0225	0·1151	0·6308

OC = Oxygen consumption (ml./min.).
W = Body weight (kg.).
T = Room temperature (° C.).
V = Ventilation (ml./min.).

AVO = A-V O_2 difference (vols. p.c.).
OCAP = O_2 capacity of blood (vols. p.c.).
CO = Cardiac output (ml./min.).

Cardiac output. The third regression equation of Table 4 shows that the cardiac output was approximately related to Weight$^{2/3}$ (i.e., surface area). The relation to oxygen capacity had a probability of 0·07, which is considered to be significant in view of the clear cut results of the second regression equation of Table 4 and comparable results in a series of anaemic animals reported elsewhere (Darian Smith, 1954). The cardiac output was thus greater in larger animals and rose with a fall in haemoglobin and a fall in room temperature. No significant relation between ventilation and cardiac output was obtained.

Posture.

The cardiac output was determined in 16 recumbent animals. The mean cardiac output was 428 ml./min. (S.D. 14; S.E. of mean 4·4). The suspended animals, when allowing for the difference in weight, room temperature and oxygen capacity, had a mean cardiac output of 413 ml./min. (S.D. 16; S.E. of mean 2·3). There was thus a considerable overlap between the two groups. Whilst the difference in mean values was statistically significant, it was so slight as to be probably biologically unimportant (c.f. Donald *et al.*, 1953).

Effect of light anaesthesia.

This was studied in two small groups of rabbits. The first group (4 rabbits, average weight 1·7 kg.) was catheterized under nembutal and anaesthesia maintained for 2 hours following operation (dose 60 mg./2 hr.). The cardiac output was then measured and the animals were allowed to recover from the anaesthetic. A control cardiac output was determined four hours later. The second group (4 rabbits, average weight 1·8 kg.) was catheterized under sodium pentothal. The animals were then allowed to recover from the anaesthetic and a control cardiac output done 3 hours after catheterization. They were then anaesthetized with intravenous nembutal over a period of 2 hours (dose 60 mg./2 hr.) and then the cardiac output was again determined. In this whole group of experiments the cardiac output fell from 389 ml./min. to 322 ml./min. under anaesthesia which, however, was not significant (S.E. of the difference 55·0, $p=0·3$). The order in which the anaesthetic was given was not significant. The oxygen consumption in the control determinations was 26 ml./min. and fell to 20 ml./min. after 2 hours anaesthesia. This was significant (S.E. of difference=2·3, $p=0·04$). The ventilation was reduced from 970 ml./min. to 650 ml./min. under these conditions. There was no evidence of arterial unsaturation with the dose of anaesthetic used, the percentage saturation remaining at 97 p.c. during both control and anaesthetic periods. The arterial CO_2 content rose from an average value of 41 volumes p.c. to 46 volumes p.c. under anaesthesia.

DISCUSSION.

Errors in cardiac output determination.

These fall into three possible categories: (i) analytical errors, (ii) inappropriate application of the Fick principle (e.g., incorrect positioning of the cardiac

catheter), (iii) physiological fluctuations. The analytical errors in determining oxygen consumption are approximately 2 p.c. The analytical error in determining the oxygen content in a given sample of blood is no greater than 0·2 volumes p.c., giving an error for the estimate of A-V oxygen difference of about 8-9 p.c. It follows that the maximum analytical error of an estimate of cardiac output is about 10-11 p.c.

A further possible source of error in the determination of A-V oxygen difference is the failure to obtain true mixed venous blood from the right atrium. Recent work in dogs, by Griffin, Wood and Essex (1951), has shown that the oxygen content of atrial and pulmonary arterial blood did not differ significantly or systematically from one another. Similar findings were obtained in man for right atrial and ventricular blood by Cournand, Riley, Breed, Baldwin and Richards (1945). These results are probably applicable to the much smaller rabbit atrium and suggest that this source of error was small and accounted for little of the residual sum of squares of the A-V oxygen difference in Table 4.

It is clear then that the standard error of single determinations of oxygen consumption, A-V oxygen difference and cardiac output are somewhat greater than maximum possible analytical error, the difference indicating the range of the physiological fluctuations of these measurements. The causes of some of these fluctuations have been analysed in Table 4 and will be considered below.

Cardiac output in normal rabbit.

The present series differs from most other work on animals in that the results from a large number of unanaesthetized animals have been systematically analysed in order to establish an adequate baseline for other work. Comparison of the results summarized in Table 3 with those of other authors using anaesthetized rabbits and cats reveals a considerable disparity. Leegaard (1926), working with rabbits anaesthetized with urethane, obtained an average value of 130 ml./kg./min. for cardiac output and about 7 ml./kg./min. for oxygen consumption. Similar figures have been obtained by Fasold and Hartl (1928), Meier, Tripod and Wirz (1949) in anaesthetized rabbits, and Baxter, Cunningham and Pearce (1952) in anaesthetized cats. The results of the present series are about 50-100 p.c. higher than the results quoted. We have noticed in this laboratory that results similar to those of the above authors are obtained when drastic operative procedures have been carried out or very deep anaesthesia used. In lightly anaesthetized animals, while the cardiac output was not markedly altered, there was a reduction in oxygen consumption of 25-30 p.c., agreeing with the work of Shackman, Graber and Redwood (1951) on the effects of anaesthesia in man. Thus anaesthetized animals used as controls preparatory to a given experimental procedure are not comparable to unanaesthetized animals, but are in a different "steady state."

Physiological causes of variation of O_2 consumption, A-V O_2 difference and cardiac output.

Some of these have been listed in Table 4. The relation of oxygen consumption to weight was almost linear (i.e., O_2 consumption=$kW^{.96}$) and apparently not related to surface area (i.e., O_2 consumption=$kW^{.67}$). This is in agreement with the work of Galvao (1947). The inverse relation of O_2 consumption to environmental temperature over a range of 15-25° C. is additional evidence that heat regulation had returned to normal after the preceding barbiturate anaesthesia (Hemingway, 1941). The lower limit of the thermal comfort zone in man in Australia is about 20-22° C. (Kellerman, 1954), and it appears that the lower limit of the comfort zone in rabbits is not far from this figure.

The statistical analysis of factors affecting the A-V O_2 difference has not previously been attempted. The relation of A-V O_2 difference to O_2 consumption suggests that small increases in the body metabolism are not *per se* accompanied by a proportional rise in cardiac output, but by a greater oxygen utilization by the tissues. The A-V O_2 difference also rose by 1 volume p.c. for a change in haemoglobin of about 3 gm. (range of haemoglobin 8-16 gm. p.c.). Thus, even with slight grades of anaemia, the cardiac output tends to be somewhat elevated.

An analysis of factors influencing cardiac output showed that the cardiac output was related to surface area ($W^{.68}$) rather than body mass directly (c.f. Cournand *et al.*, 1945). From inspection of the right half of Table 4 it may be seen that the independent variates considered account for about 70 p.c. of the total variation of oxygen consumption and for about 60 p.c. of the total variation of A-V O_2 difference. As would be expected, these variates account for only 40 p.c. of the total variation of cardiac output. The surface area accounts for only about 20 p.c. of the total variation. While the results show a very definite relation of cardiac output to some function of weight, this accounts for only a small fraction of the total variation. The practice of reporting cardiac output/unit weight, although commonly done in animal work and previously done in this laboratory, is therefore not warranted. The same criticism probably applies to the almost universal practice of reporting human cardiac output figures per unit surface area. It has frequently been pointed out by statistical authorities that such *a priori* corrections are of little real value and may even lead to erroneous conclusions (Fisher, 1947).

The mean arterial carbon dioxide content of these animals, 44 volumes/100 volumes of blood, was somewhat lower than that reported in dogs (Dill, Edwards, Florkin and Campbell, 1932) and in man (Cournand *et al.*, 1945), but agreed with Mellanby's (1922) results in rabbits. The significance of these results was not obvious.

SUMMARY.

The mean cardiac output in 55 unanaesthetized rabbits was 415 ml./min. and the oxygen consumption was 25 ml./min., the average weight being 1·9 kg. Data of the fiducial probability limits of the cardiorespiratory measurements are presented. There was negligible difference in cardiac output between a group of suspended and a group of recumbent animals.

Light barbiturate anaesthesia did not produce a significant change in cardiac output, but led to a drop in oxygen consumption and ventilation.

Some of the physiological causes of fluctuations in oxygen consumption, A-V oxygen difference and cardiac output are analysed.

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THE CARDIAC OUTPUT IN ACUTE ANOXIA IN UNANAESTHETIZED RABBITS[1]

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Previous work on the effect of acute anoxia on the cardiac output is conflicting. Motley, Cournand, Werkö, Himmelstein and Dresdale (1947) reported a small drop in the cardiac output in man, but later work by the same group showed that if proper attention was paid to the attainment of "steady state" conditions in anoxia, small elevations in cardiac output occurred (Fishman, McClement, Himmelstein and Cournand, 1952; Doyle, Wilson and Warren, 1952). Westcott, Fowler, Scott, Hanenstein and McGuire (1951) did not find any significant change in cardiac output in man following short term exposure to acute hypoxia. The results of animal work are also conflicting. Doi (1921) showed that there was no change in cardiac output in anaesthetized cats; Lewis and Gorlin (1952) reported little change in the cardiac output of anaesthetized dogs, except with very severe degrees of anoxia, when the cardiac output rose. Harrison and Blalock (1927) obtained a rise in cardiac output in both narcotized and unanaesthetized dogs with severe degrees of anoxia. Courtice and Korner (1952) found a reduction of the cardiac output during anoxia in a small series of unanaesthetized rabbits. Recently the results of a large series of cardiac output determinations in normal unanaesthetized rabbits have been published and the causes of some of the physiological fluctuations in cardiac output, oxygen consumption and A-V oxygen difference analysed (Korner and Darian Smith, 1954). In view of these conflicting results, the earlier work was extended. In the present experiments special care was taken (i) to allow an adequate time of exposure to a given low oxygen mixture to ensure the attainment of a "steady state," (ii) to examine the cardiovascular and respiratory effects of a range of oxygen mixtures from 21 p.c. to 11 p.c. oxygen, (iii) to test the ability of the rabbits to recover from the effects of anoxia.

METHODS.

Thirty rabbits varying in weight from 1·3 to 3·0 kg. were used. All animals were unanaesthetized. The method of catheterization, of suspending the animals and analytical methods used are fully described elsewhere (Korner and Darian Smith, 1954).

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All gas mixtures (21 p.c. O_2, 18 p.c. O_2, 15 p.c. O_2, 12 p.c. O_2, 11·5 p.c. O_2) were supplied from specially prepared gas cylinders. An open-circuit system of gas exchange was employed and the animal breathed the required mixture through valves, from an anaesthetic bag, which was kept half filled by regulating the valves of the cylinder.

RESULTS.

The effect of breathing different oxygen mixtures on the cardiac output.

A factorial experiment was designed to test the effect of different oxygen mixtures on cardiac output, oxygen consumption, etc. After each rabbit had recovered from the cardiac catheterization, it was suspended, the respiratory valves attached, and the animal breathed room air for half an hour. At the end of that time the control cardiac output was determined. Following this, the animal breathed the gas mixture whose effect was to be tested. The mixtures used were 21 p.c., 18·3 p.c., 15 p.c., 11·5 p.c. oxygen in nitrogen. Each animal was tested only on one mixture. Four animals breathed each type of oxygen mixture, two of them for one hour and two for four hours. At the end of this time a second cardiac output determination was carried out. The results are summarized in Table 1 and have been analysed statistically, using the analysis of variance technique. With the exception of the animals breathing 18·3 p.c. oxygen, the changes in cardiac output and oxygen consumption were identical in the 1 and 4-hour subgroups on each mixture. The changes in cardiac output are shown in Fig. 1. There was a slight, but not significant, rise in cardiac output when the animals breathed 21 p.c. oxygen. The 1-hour subgroup, breathing 18·3 p.c. oxygen, showed a slight depression in cardiac output, but the latter had returned to normal after 4 hours. There was a significant drop in cardiac output of about 30 p.c. in animals breathing 15 p.c. and 11·5 p.c. oxygen. Table 1 shows that there was a significant reduction in the oxygen consumption/min. in the last two groups of animals with only slight changes in the R.Q. As will be seen later, reduction in the oxygen consumption is not an invariable finding following exposure to low oxygen mixtures. In rabbits, the cardiac output has, however, always been depressed even when the oxygen consumption was normal. There was no significant change in mean arterial pressure or heart rate in any of the experiments. The response of the right atrial pressure was variable. There was a rise in the right atrial pressure in some animals with 15 p.c. and 11·5 p.c. oxygen, but its significance was doubtful.

Analysis of factors influencing oxygen consumption, A-V oxygen difference and cardiac output in animals breathing 18·3 p.c., 15 p.c., 12 p.c., 11·5 p.c. oxygen in nitrogen.

Oxygen consumption. The effects of the oxygen concentration in the inspired gas mixture, the weight of the animal and the room temperature on the oxygen consumption/minute are summarized in the first multiple regression

TABLE 1.

Cardiorespiratory effects of various oxygen mixtures.

Group	Sub-group	Weight kg.	Cardiac output ml./min.		Arterial pressure mm. Hg.		Right atrial pressure cm. H_2O		Arterial saturation p.c.		Oxygen consumption ml./min.		Ventilation litres/min.		R.Q.	
			T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
A	1	2·7	529	698	136	148	6	6	98	97	38·9	48·2	1·3	2·6	0·80	0·70
		2·3	459	423	130	125	4	5	95	94	36·1	34·7	3·3	2·6	1·0	0·79
	2	2·1	422	482	128	148	4	5	97	96	31·7	40·5	1·1	2·1	0·79	0·69
		1·6	307	338	114	114	6	5	97	98	30·7	31·5	1·6	1·2	0·77	0·76
B	1	2·0	339	233	132	142	2	2	97	93	21·3	18·7	1·6	2·2	0·87	1·0
		1·9	398	348	152	142	2	0	99	91	22·6	24·4	1·0	1·3	0·90	0·99
	2	1·8	387	337	140	140	8	7	98	91	22·1	19·3	1·0	1·0	0·67	0·72
		1·8	369	382	148	154	7	8	98	92	29·5	24·4	1·4	1·0	0·83	0·88
C	1	2·2	339	246	152	144	7	14	98	88	35·3	26·2	1·7	1·7	0·83	0·90
		2·0	281	187	152	152	8	13	97	78	27·1	21·4	1·6	1·6	0·90	1·10
	2	2·1	392	225	136	140	3	0	96	89	26·2	17·7	1·1	2·0	0·78	0·74
		2·0	340	238	150	152	4	6	96	81	23·5	19·8	1·0	1·7	0·83	0·91
D	1	2·7	416	315	126	126	5	7	98	81	35·7	19·6	1·8	1·9	0·78	0·90
		2·2	419	337	130	150	5	0	98	48	28·7	12·6	1·1	2·3	0·92	0·87
	2	3·0	501	324	148	144	0	12	97	80	33·6	24·3	1·1	2·5	0·78	0·83
		2·6	547	358	140	140	4	11	98	85	31·0	21·9	1·0	2·3	0·75	1·0

Group A=Breathing 21 p.c. oxygen.
Group B=Breathing 18·3 p.c. oxygen.
Group C=Breathing 15 p.c. oxygen.
Group D=Breathing 11·5 p.c. oxygen.

T_1=Time of control determination, breathing room air.
T_2=Time of second determination, breathing test mixture.
Subgroup 1=Breathing test mixture for 1 hour.
Subgroup 2=Breathing test mixture for 4 hours.

equation of Table 2. In the group of 22 animals considered in the analysis, the oxygen consumption/minute fell as the oxygen concentration in the inspired gas mixture was reduced, and was significantly related to body weight. The room temperature within the limits of the experiment (15-25° C.) did not exert a significant effect on oxygen consumption, in contrast to the findings with normal rabbits. The results are illustrated graphically in Fig. 2.

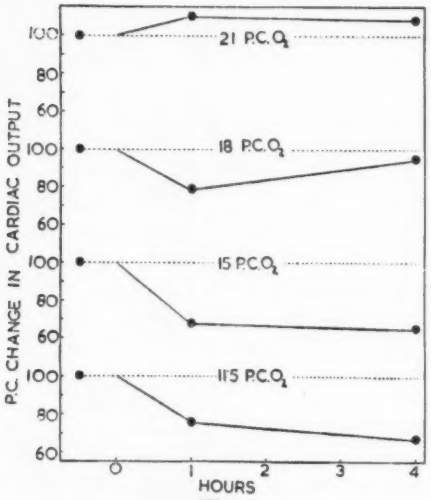

Fig. 1.

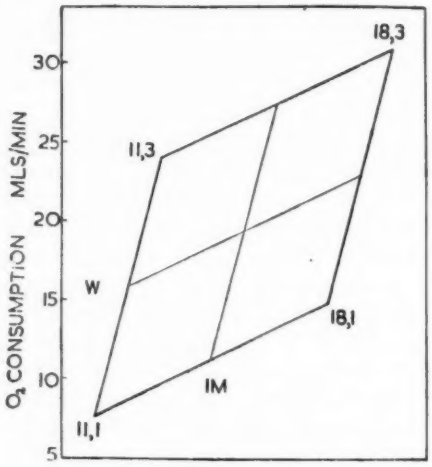

Fig. 2.

Fig. 1. Changes in cardiac output on breathing various O_2-N_2 mixtures. Gas mixtures breathed from 0-4 hours.

●···· Control level.
—●— Cardiac output when breathing test mixture.

Fig. 2. Nomogram illustrating relation between O_2 consumption (ml./min. at STP), inspired O_2 concentration (IM) and body weight (W). The coordinates of each point are expressed in the order IM, W. Range of gas mixtures breathed 11-18 p.c.

A-V oxygen difference. There was a significant relationship (Equation 2, Table 2) between the A-V oxygen difference and arterial oxygen content. The A-V oxygen difference decreased on the average by 0·3 volumes p.c. for a drop of 1 volume p.c. in arterial oxygen content. In contrast to the findings with normal rabbits breathing air there was no significant relation between the oxygen consumption/minute and the A-V oxygen difference.

Cardiac output. The cardiac output in the anoxic animals was significantly related to the arterial percentage saturation and to the body weight (Equation 3, Table 2). The cardiac output fell on the average 30 ml./min. (12 p.c. of mean) for a drop in the percentage arterial saturation of 10 p.c. (range of arterial saturation in anoxic animals 93-48 p.c.). These results are shown graphically in Fig. 3.

TABLE 2.

Factors affecting oxygen consumption, A-V oxygen difference and cardiac output in anoxia (breathing 18·3—11·2 p.c. O_2).

	No. of results	Multiple regression equation	First partial regression coefficient	Second partial regression coefficient	Third partial regression coefficient
Oxygen consumption	22	OC=17·6+0·96 (IM—13·5)+7·9 (W—1·9)	0·96±0·38 p=0·02	7·9±2·2 p=0·001	—
A-V O_2 difference	22	AVO=6·7+0·035 (OC—17·6)—0·71 (W—1·9) +0·29 (OCONT—12·3)	0·35±0·061 not significant	—0·71±0·65 not significant	0·29±0·12 p=0·02
Cardiac output	22	CO=247+92 (W—1·9)+3·0 (AS—75)	92±32 p=0·01	3·0±0·94 p=0·005	—

OC=Oxygen consumption (ml./min.).
IM=Oxygen concentration in inspired gas mixture, p.c.
W=Weight, kg.
AVO=Arteriovenous oxygen difference, vols. p.c.
OCONT=Arterial oxygen content, vols. p.c.
CO=Cardiac output, ml./min.
AS=Arterial percentage saturation.

The response of cardiac output and oxygen consumption to a given inspired gas mixture.

It is important to assess the variation in response of cardiac output and oxygen consumption when breathing a given inspired gas mixture. The results

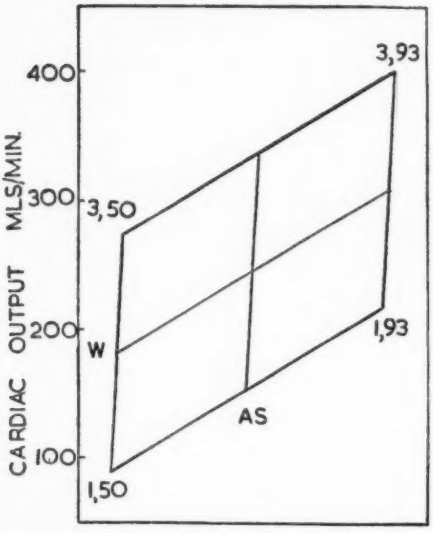

Fig. 3. Nomogram illustrating relation between cardiac output (ml./min.), body weight (W) and arterial oxygen saturation (AS). The coordinates of each point are expressed in the order W, AS.

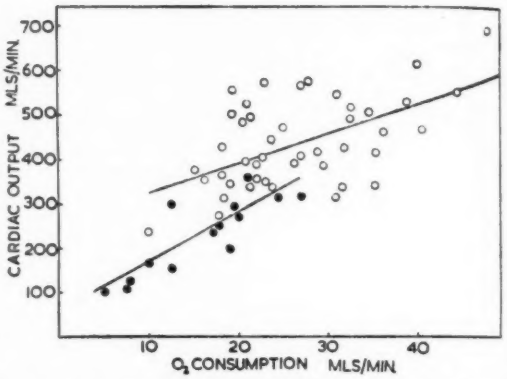

Fig. 4. Relation between cardiac output (ml./min.) to O_2 consumption (ml./min.).

○ Control animals breathing room air.
● Animals breathing 11·5-12 p.c. O_2 in N_2 for 1-4 hrs.

of a larger series of animals, eight of which breathed 11·5 p.c. oxygen and six of which breathed 12 p.c. oxygen, have therefore been analysed. For the purposes of this analysis the inspired gas mixture may be considered to be practically uniform for the whole series. It was noted that the arterial saturation in animals breathing a given mixture for 1 to 4 hours showed considerable individual variation, from 48 p.c. to 87 p.c., this corresponding to about 25 mm. Hg. difference in arterial O_2 tension. The cardiac output in these animals behaved as shown in Fig. 3. Further, the following multiple regression equation expresses the relation of arterial oxygen saturation to the oxygen consumption of the animal:

$$\text{Art. Sat.} = 70{\cdot}3 + 1{\cdot}86\ (\text{OC} - 15{\cdot}4) - 0{\cdot}5\ (\text{W} - 1{\cdot}8)$$

where the symbols are the same as in Table 2.

The first regression coefficient only was statistically significant ($p = 0{\cdot}001$).

Thus in animals where the oxygen consumption was greatest, the percentage arterial saturation, and thus the cardiac output was highest. The significance of this will be considered below. Of interest also from the point of view of behaviour of cardiac output when breathing a single low oxygen mixture are the results shown in Fig. 4, where the cardiac output has been plotted against the oxygen consumption in a large series of normal animals and in animals breathing 11·5 to 12 p.c. oxygen. It may be seen from the regression lines calculated from the data that the cardiac output at that level of anoxia is significantly lower than would be expected merely from the reduction in oxygen consumption, especially with the lower oxygen consumptions.

"Steady-state" conditions in anoxia. The results of duplicate determinations of oxygen consumption 15 minutes prior to cardiac output determination when breathing different low oxygen mixtures from 1 to 4 hours are shown in Table 3. The results of these duplicate determinations are quite comparable with similar duplicate estimations on normal animals (Korner and Darian Smith, 1954) and show that within the above time limits the rabbits were in a reasonably steady state.

TABLE 3.

Results of duplicate determinations of oxygen consumption (ml./min.) in anoxia.

	T_1	T_2
	25·2	26·7
	13·8	12·3
	15·9	16·9
	19·0	20·1
	6·7	7·5
	16·6	17·6
	24·4	25·6
	21·9	21·3
	16·0	15·6
	9·9	9·5
	12·4	13·2
	14·1	13·6
Mean	16·3	16·7

T_1=Time of first determination.
T_2=15 minutes after T_1.

Recovery from breathing 12 p.c. oxygen.

In this experiment 6 rabbits were used. After recovering from the cardiac catheterization, a control determination of oxygen consumption was made and repeated half an hour later. Three of the rabbits were allowed to breath 12 p.c. oxygen for 1 hour and three others for 2 hours. At the end of that time the cardiac output was measured and the animals immediately afterwards allowed to breath room air. After 2½ hours the cardiac output was again determined.

The results are shown in Table 4. The cardiac output rose on the average by about 30 p.c. after reverting to air. Recovery to normal occurred in five out of six animals. In three out of the six animals there was little change in the oxygen consumption, though all showed some depression of the cardiac output. On the average there was a slight depression in the oxygen consumption during exposure to the gas mixture. While breathing 12 p.c. oxygen the respiratory minute volume doubled and there was a slight rise of the R.Q. When the animals were allowed to breath room air again, the R.Q. quickly returned to normal. The ventilation returned to the control value somewhat more slowly. There was a significant rise in oxygen consumption ($p=0{\cdot}01$) above the initial control value 1 hour after reverting to room air, but after $2\frac{1}{2}$ hours the oxygen consumption had returned to normal.

TABLE 4.

Cardiorespiratory changes following anoxia.

		T_1	T_2	T_3	T_4
Cardiac output (ml./min.)	A	—	321	—	410
		—	153	—	362
		—	245	—	359
	B	—	276	—	328
		—	123	—	100
		—	260	—	350
Oxygen consumption (ml./min.)	A	21·4	25·6	32·9	22·5
		17·2	12·3	26·4	21·0
		20·2	15·0	21·0	16·3
	B	19·6	20·1	20·7	19·9
		18·7	7·5	21·9	12·0
		17·5	17·6	22·0	19·4
Ventilation (ml./min.)	A	875	1990	1173	880
		859	1856	1692	1179
		1122	2118	1568	1614
	B	823	1560	807	780
		823	1388	1364	1396
		674	1360	663	646
R.Q.	A	0·88	1·08	0·98	0·98
		1·0	1·2	1·0	1·0
		0·77	1·0	1·0	0·71
	B	0·87	0·88	0·70	0·86
		0·82	0·99	0·75	0·87
		0·84	1·0	0·80	0·87

T_1=Control, breathing air.
T_2=End of period of breathing 12 p.c. oxygen.
T_3=1 hour after returning to air.
T_4=$2\frac{1}{2}$ hours after returning to air.
A=Breathing 12 p.c. oxygen for 1 hour.
B=Breathing 12 p.c. oxygen for 2 hours.

Changes in arterial CO_2 content.

There was no significant change in CO_2 content when the rabbits were breathing 21 p.c. oxygen. There was a fall of 3 to 5 volumes p.c. on breathing 18 p.c. oxygen and a fall of 5 to 8 volumes p.c. with the lower oxygen mixtures.

DISCUSSION.

The application of the Fick principle to the present experiments.

In a recent paper Fishman and his associates (1952) stressed the necessity for achieving a "steady state" between the air and blood gas phases before applying the Fick principle to the determination of cardiac output in men acutely exposed to low oxygen mixtures. These workers defined the following criteria as satisfying their steady state requirements: (i) Emotional stability; (ii) constancy of oxygen consumption compared to that when breathing air; (iii) constancy of the R.Q. In man there is evidence that the oxygen consumption is not altered during exposure to low oxygen mixtures or high altitudes (D'Angelo, 1946; Houston and Riley, 1947; Rahn and Otis, 1949). When small animals are acutely exposed to anoxia their metabolism is usually depressed (Blood, Elliot and d'Amour, 1946; Lintzel, 1931; Kottke, Phalen, Taylor, Visscher and Evans, 1948). To ensure that steady state conditions should be attained in the present experiments, emphasis was placed on the following points: (i) unanaesthetized animals only were used; (ii) longer periods of exposure to low oxygen mixtures than used in most human work were allowed to ensure maximum equilibrium between blood and air gas phases; (iii) in many cases oxygen consumption and R.Q. were measured 15 minutes before doing the actual cardiac output determination (Table 3) and showed excellent agreement. The R.Q. rose a little as a result of the overall hyper-ventilation produced by the anoxia. The change in R.Q. was well within the limits defined by Fishman *et al.* in the main series of the experiments (Table 1), but was slightly more elevated in the "recovery experiment" series (Table 4). Courtice and Korner (1952) showed that the arterial oxygen saturation was practically the same for rabbits breathing 11 p.c. O_2 for periods from 20 minutes after exposure to the gas mixture up to 5 hours after exposure. It follows that the animals in this series were in a steady state and that the application of Fick's principle is quite valid under the conditions of the present experiments.

Oxygen consumption in anoxia.

The results are shown graphically in Fig. 2. A comparable figure for normal animals breathing air is published elsewhere (see Korner and Darian Smith, 1954, Fig. 4). In normal rabbits the oxygen consumption rose as the room temperature fell from 25 to 15° C. In anoxic rabbits the ability to raise their metabolism in response to cold was lost, in agreement with the results of

other workers (Kottke *et al.*, 1948; Hemingway and Nahas, 1952). As may be seen from Fig. 2, the average oxygen consumption of a rabbit weighing 2 kg. and breathing 11·5 p.c. oxygen was about 17 ml./min. with a room temperature range of 15 to 25° C. In animals of the same weight breathing air, the oxygen consumption at 15° C. was about 30 ml./min.; at 25° C. the average oxygen consumption was only about 24 ml./min. There was thus a much greater difference between the anoxic and normal rabbits at low room temperatures, and this difference probably depends on failure of the normal heat regulating mechanism during anoxia. The room temperature at which the experiment is carried out is thus of importance in determining whether an animal will have a large or small fall in oxygen consumption. This may partly account for the fact that three animals in the "recovery series" showed no fall in oxygen consumption. Of great interest were the results obtained in the recovery experiments following reversion to room air. The oxygen consumption rose significantly for the first hour following anoxia, suggestive of repayment of an oxygen debt.

Cardiac output in anoxia.

The lethal limit of anoxia in suspended, unanaesthetized rabbits was about 9·8 p.c. inspired oxygen; of three animals exposed to this mixture none survived for more than 2½ hours. Rabbits thus seem to be more susceptible to anoxia than cats and dogs under anaesthesia (c.f. Doi, 1921; Lewis and Gorlin, 1952). Acute anoxia (11·5 to 15 p.c. oxygen) in the present experiments produced "low output" heart failure, together with some systemic vasoconstriction. The cardiac output was most depressed with the lowest arterial percentage saturations; the latter, in turn, depended on the efficiency of the respiratory response to anoxia. The animals that had the highest oxygen consumption for a given ventilation had the highest arterial saturation. These results suggest that in animals with a ventilatory response of maximal efficiency, the ventilation/perfusion ratio of different parts of the lungs was more uniform than in animals which failed to show maximal respiratory adaptation to acute anoxia. If the ventilation/perfusion ratio varied greatly in different areas of the lung, a considerable amount of the mixed venous blood would be insufficiently aerated, thus lowering the arterial percentage saturations. The arterial oxygen tension thus seems to be very important in regulating the cardiac output in rabbits. The depressant effect with low arterial saturations is probably a direct one on the myocardium owing to an insufficient pressure head of oxygen to maintain the normal myocardial requirements. It was seen in Fig. 4 that the cardiac output was depressed to a greater extent on the average than could be accounted for by the general depression in metabolism. The rapid return to normal of the cardiac output in five out of six animals following 1 to 2 hours exposure to 12 p.c. oxygen shows that the myocardium does not become permanently damaged. Apparently the cardiac failure induced by the anoxia is rapidly reversed when sufficient oxygen is again available.

Other work on cardiac output changes in anoxia.

The results of the present experiments on rabbits differ from the results reported in other species. In man, Fishman, *et al.* (1952) have shown that the cardiac output rises slightly in acute anoxia. Similar work in unanaesthetized dogs by Harrison and Blalock (1927) seemed to agree with the work in man. In the experiments of Harrison and Blalock no particular care was taken to reach steady state conditions; it is, in fact, likely that the Fick principle was inappropriately applied to their results, as their animals were rebreathing a gas mixture in which the oxygen concentration was continuously falling. Work on other animals (c.f. Lewis and Gorlin, 1952) was done on anaesthetized animals, where the arterial saturation of the control blood was mostly low (80 to 85 p.c.). Korner and Darian Smith (1954) have shown that even light anaesthesia depresses the metabolism of normal rabbits, and Hemingway (1941) has shown that barbiturate anaesthesia depresses the heat regulation mechanism of animals. It is therefore unlikely that a true picture of the effects of anoxia on cardiac output is obtained in anaesthetized animals, as these are in quite a different cardiavascular, respiratory and metabolic state from unanaesthetized animals. It seems then that the present results establish a species difference between rabbits and man. The results with 18 p.c. oxygen showed that after a slight temporary depression, the cardiac output returned to normal after 4 hours. With the more severe degrees of anoxia the cardiac output was still depressed after 4 hours. The species differences in response to anoxia may depend on the rate of adaptation or acclimatization to anoxia, which seems to be most effectively developed in man.

SUMMARY.

The cardiac output was depressed by about 30 p.c. in unanaesthetized rabbits when exposed to 15-11·5 p.c. oxygen in nitrogen for periods from 1 to 4 hours. Breathing 18 p.c. oxygen had only a slight and transient effect on the cardiac output. None of the hypoxic mixtures produced a significant change in heart rate or mean arterial pressure. There was a suggestion of some rise in right atrial pressure with 15 and 11·5 p.c. oxygen.

There was a depression of oxygen consumption in the rabbits on exposure to the hypoxic mixtures. Evidence was presented that anoxia disturbs the normal heat regulating mechanism of unanaesthetized rabbits.

The depression of the cardiac output was thought to be due to a direct effect of anoxia on the myocardium; it was rapidly reversible in 5 of 6 animals tested on returning to 21 p.c. oxygen.

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THE CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF ORGANIC ACIDS AND THEIR APPLICATION TO YEAST

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The necessity for separating organic acids of biological interest, particularly for studies involving radioactive isotopes, has been met in recent years by the development of quantitative silica gel or Celite column chromatography. It was soon realised (Isherwood, 1946) that separation of complex acid mixtures could not be obtained on one column with one solvent system of fixed composition.

Bulen, Varner and Burrell (1952) used more than one column, developed with solvents differing in composition and in the proportion of their constituents, to separate the organic acids of plant tissues. Phares *et al.* (1952) isolated many biosynthetic organic acids and several inorganic acids by similar methods. Several systems have been proposed in which only the one column is employed, but the solvent system is increased in polarity in a number of discrete steps throughout the run. Marvel and Rands (1950) separated many organic acids in this way, but did not include some of the more commonly occurring acids of biological interest. Frohman, Orten and Smith (1951) developed a system whereby all the acids of the Krebs cycle could be separated and estimated quantitatively in animal tissues.

The idea was further developed by Donaldson, Tulane and Marshall (1952), who designed an apparatus for gradually and automatically increasing the polarity of the solvent throughout the run. In the present work, the latter technique was extended to the quantitative separation of several additional acids and applied to an investigation of the organic acids in yeast. Two-dimensional paper chromatography was used to identify the individual acid fractions obtained.

EXPERIMENTAL.

Column chromatography.

Apparatus and reagents. Silica gel was prepared according to Isherwood (1946). The apparatus used was identical with that of Donaldson *et al.* (1952), the solvent reservoir being subjected to pressure from a nitrogen cylinder to maintain a constant rate of flow of solvent throughout the experiment. The solvent system, size of column, and amounts of silica gel and aqueous phase used were the same as those described by Marshall, Donaldson and Friedberg (1952).

The acids investigated were acetic, succinic (B.D.H., Analar), fumaric, glycollic, maleic, malonic, tartaric (B.D.H., Laboratory Reagent), α-ketoglutaric, L-malic, tricarballylic (Light's), citric (Baker's A.R.), glutaric (Hopkins and Williams), lactic (Judex, A.R.), formic (F. H. Faulding), oxaloacetic, pyruvic and *cis*-aconitic. The oxaloacetic acid was prepared from sodium diethyloxaloacetate (Krampitz and Werkman, 1941) and was shown to be pure by the aniline citrate method (Edson, 1935). Pyruvic was freshly distilled *in vacuo* and kept as a molar, unneutralized solution. *Cis*-aconitic acid was prepared from *trans*-aconitic acid (Malachowski and Maslowski, 1928).

The silica gel column was supported by a chromatographic tube, 35 cm.×6 mm., having one end bevelled and constricted to a diameter of 2 mm. The effluent was cut into fractions by an automatic fraction collector operating on a time basis.

Preparation of acids for the column. All acids applied to the column, whether as standards to determine their order of elution or in yeast extracts, were dissolved in a 40 p.c. *n*-butanol-60 p.c. chloroform mixture (Marvel and Rands, 1950). The filtrate of a boiled 1:1 aqueous yeast extract (Effront Yeast Co., Victoria) was acidified to pH 1 with 5 N sulphuric acid and 3 ml. pipetted on to 25 gm. anhydrous sodium sulphate in a mortar, mixed thoroughly, transferred quantitatively to a Soxhlet thimble and extracted with freshly distilled anaesthetic ether for six hours. The ethereal extract was evaporated to dryness at room temperature by a current of air, the residue dissolved in the chloroform-butanol mixture and the appropriate amount (usually equivalent to 0·5 gm. wet weight of yeast) applied to the column.

Procedure. 1·1 gm. silica gel was added directly to 0·8 ml. 0·05 N sulphuric acid, mixed thoroughly and sieved (85 mesh). Chloroform was added until a lump-free slurry was obtained and this was poured into the chromatographic tube, which had at the constricted end a filter paper disc to support the column. 10 ml. chloroform were passed through the column under a pressure of 3 lb./sq. inch, taking care not to allow the chloroform level to fall below the level of the gel. The column was gently tamped down with a glass rod, excess chloroform removed and the appropriate amount of sample was added. This was allowed to pass into the column under slight pressure, again avoiding drying out the top of the gel. The column was then connected to the apparatus containing the developing solvent. The solvent reservoir was charged with a 1:1 mixture of 30 p.c. *n*-amyl alcohol and 30 p.c. *tert*-amyl alcohol in chloroform, which was delivered into the mixing vessel originally containing 50 ml. chloroform. 220 one-minute, followed by two-minute fractions, were collected, the pressure being adjusted to give a rate of flow of 22 drops/minute (≡ 0·3 ml.). One drop 0·02 p.c. phenol red was added and each fraction was titrated with caustic soda, the tube contents being vigorously mixed by a stream of CO_2-free air. The fractions containing the individual neutralized acids were pooled.

Paper chromatography.

Both ascending and descending chromatograms were run.

Apparatus. Whatman No. 1 filter paper was used throughout. For descending chromatograms, drain pipes similar to those of Consden, Gordon and Martin (1944) were used as chromatographic chambers. The pipes stood in close-fitting trays to which they were sealed by pitch. Weighted glass plates rested firmly on the top of the pipes, making the chambers air-tight. Papers 6 in. × 24 in. were suspended from stainless steel troughs containing the developing solvent, and run in the conventional manner. Ascending chromatograms were run in battery jars, the tops of which were also sealed with weighted glass plates. Papers 12 in. × 12 in. were stapled to form cylinders (Stark, Goodban and Owens, 1951) and stood in Petri dishes. The developing solvents were poured into the troughs or dishes through a small hole drilled in the centre of the glass plate.

Solvents. (1) Alkaline solvent: 8 vol. 95 p.c. ethyl alcohol, 1 vol. water, 1 vol. 7·5 N ammonium hydroxide. (2) Acidic solvent: water-poor phase from 3 vol. *n*-butyl alcohol, 6 vol. *iso*-butyl alcohol, 9 vol. water, 3 vol. formic acid (90 p.c.). Both phases of the acidic solvent remained in mutual contact with frequent shaking for eight hours before use. The choice of solvents was made after a wide range of described and new solvent mixtures had been investigated by the capillary ascent test-tube method of Rockland and Dunn (1949).

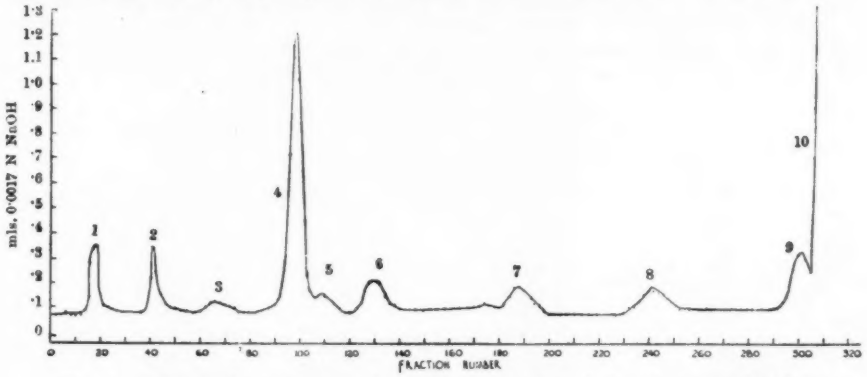

Fig. 1. Column chromatogram of yeast extract. 1 and 2, unknown acid giving a barely visible spot on a paper chromatogram (Fig. 4A, No. 7); 3, fumaric acid; 4, succinic acid; 5, α-ketoglutaric acid; 6, unknown acid running as a monocarboxylic acid in the alkaline solvent; 7, unknown acid running as a dicarboxylic acid in the alkaline solvent; 8, malic acid; 9, citric acid; 10, sulphuric acid eluted off column.

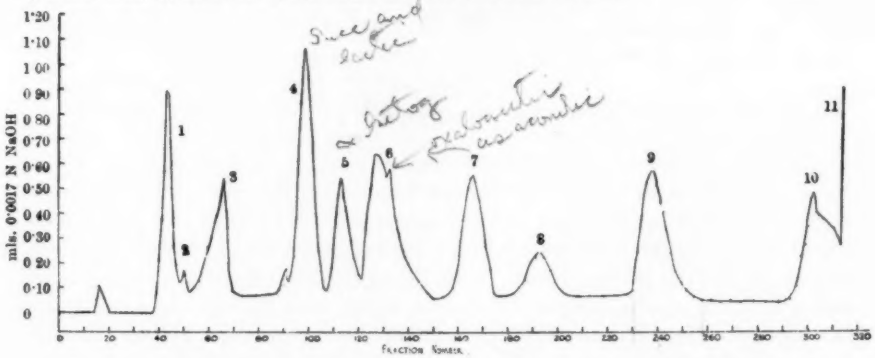

Fig. 2. Column chromatogram of a standard acid mixture. 1, acetic acid; 2, lactyl lactic acid; 3, fumaric acid; 4, succinic and lactic acids; 5, α-ketoglutaric acid; 6, oxaloacetic and *cis*-aconitic acids; 7, glycollic acid; 8, tricarballylic acid; 9, L-malic acid; 10, citric acid; 11, sulphuric acid eluted off column.

Procedure. A total volume of 6 μl. of 0·1 N aqueous solutions of standard acids was applied to the paper in a succession of spots, 4 mm. in diameter, each spot being dried with a hot air blower before the next was applied. To identify the acids from the column, the pooled fractions of each were evaporated to dryness at 70° C., air being drawn continuously over their surfaces. The sodium salts of the acids were then applied to the paper. Irrespective

of whether the chromatogram was 1- or 2-dimensional, the papers were equilibrated with the solvent system (with both phases of the acidic solvent) for at least 8 hours and usually overnight before the developing phase was added. Careful equilibration was essential for good chromatograms. To remove the acidic solvent after development, the papers were suspended in an oven, the bottom of which was covered with a tray containing water. A fan and an electric heater created a very moist atmosphere in the oven, and the temperature was kept below 50° C. After 20 minutes, fan and heater were turned off and the papers allowed to remain in the oven for a further 30 minutes. Only the fan was necessary (30 minutes) to remove the alkaline solvent completely from the papers. The spray used was 0·04 p.c. bromo-cresol green in 95 p.c. alcohol, brought to pH 6 with NaOH.

TABLE 1.

Position of acids eluted from column.

Acid	Mean fraction number
Acetic	43
Lactyl-lactic	50
Glutaric	54
Pyruvic	54
Formic	59
Fumaric	65
Lactic	100
Succinic	100
Maleic	110
α-Ketoglutaric	115
Malonic	124
cis-Aconitic	130
Oxaloacetic	133
Glycollic	167
Tricarballylic	190
L-Malic	242
Citric	303

RESULTS.

Typical column chromatograms obtained with a yeast extract and with a standard acid mixture are shown in Figs. 1 and 2 respectively. All the standard acids were identified by paper chromatography, except formic and acetic acids. These, being volatile, were removed with the swamping acid of the acidic solvent during the drying of the papers. Volatile acids were identified by their established location on the column. The acids which were run on the column, and the corresponding average fraction number which gave the highest titration figure, are listed in Table 1. The fraction at which an acid is eluted from a column is only a pointer to its identity when unknown extracts are investigated. This is demonstrated in Figs. 1 and 2. Two acids in the yeast extract (Fig. 1, Nos. 6 and 7) ran identically with oxaloacetic acid and tricarballylic acid respectively on columns prepared and run under the same conditions. In fact, pooled fractions from the columns of these yeast acids, after development on paper chromatograms run in the acidic solvent, gave spots again located in the same positions as oxaloacetic and tricarballylic acids (Fig. 4A, Nos. 3 and 5). How-

ever, in the alkaline solvent (where the R_F values of the various acids are markedly different from those in the acidic solvent) the acid in yeast which appeared to be identical with oxaloacetic ran as a monocarboxylic acid, and that apparently identical with tricarballylic acid as a fast moving diacarboxylic acid. These two acids in the yeast are as yet unidentified.

The percentage recovery of some of the acids run on the column is shown in Table 2.

TABLE 2.

Recovery of acids run on the column.

Acid	Added (μg.)	Recovered (μg.) (calculated from titration figures)	Percentage recovery
Acetic	620	625	101
cis-Aconitic	244	245	100
Citric	260	253	97
Formic	831	793	95
Fumaric	241	244	101
Glycollic	320	326	102
α-Ketoglutaric	292	257	88
L-Malic	282	286	101
Oxaloacetic	264	216	82
Pyruvic	748	688	92
Succinic	248	250	101

The lower recovery of pyruvic, oxaloacetic and α-ketoglutaric acids is probably due to their instability. In the case of α-ketoglutaric acid, the error was accentuated by a 5 p.c. impurity of succinic acid, as measured manometrically with succinoxidase (Krebs, 1937).

Different yeast samples yielded varying amounts of acids per unit weight of yeast. This variation was particularly marked with succinic acid (Table 3).

TABLE 3.

Variation of acid content in different yeast samples.

Acid	μ equiv. acid per gm. wet weight yeast			
	Sample 1	Sample 2	Sample 3	Sample 4
Fumaric	0·82	0·91	2·27	1·18
Succinic	10·9	34·4	1·38	17·9
α-Ketoglutaric	0·40	0·91	3·40	1·64
Unknown (Fig. 1, No. 6)	10·8	2·97	7·55	3·08
Unknown (Fig. 1, No. 7)	3·6	2·74	3·08	2·79
Malic	2·26	3·78	10·4	3·38
Citric	—	—	—	6.50

Although citric acid was present in all four yeast samples, it is only occasionally well separated from sulphuric acid, which is also eluted from the column at the end of the run.

A two-dimensional ascending chromatogram of a standard acid mixture is shown in Fig. 3, the alkaline solvent first separating the various acids into their classes according to the number of carboxyl groups in the molecule, and the acidic solvent permitting differentiation of the acids within their classes. The R_F values of the acids run in these two solvents are shown in Table 4.

TABLE 4.

R_F values of standard acids.

Acid	R_F values × 100	
	Alkaline solvent	Acidic solvent
cis-Aconitic	4	90
Citric	2	41
Fumaric	21	96
Glutaric	22	92
Glycollic	34	53
α-Ketoglutaric	15	65
Isocitric	2	39
Lactic	46	77
Maleic	11	59
L-Malic	12	49
Malonic	9	71
Oxaloacetic	12	56
Succinic	17	82
Tartaric	9	24
Tricarballylic	3	72

Although a two-dimensional chromatogram is necessary for more positive identification of the acids in the yeast, a one-dimensional chromatogram, developed in the acidic solvent, was sufficient to separate all the detectable acids present. Use of the two solvents gave no increase in the number of spots on the paper. The one-dimensional technique has the added advantage of being less time consuming and giving spots which are more compact and sharply defined.

One-dimensional descending chromatograms of an appropriate amount of the same yeast extract used for Fig. 1 and of a standard acid mixture are shown in Fig. 4.

DISCUSSION.

The results of the column chromatograms are essentially the same as those obtained by Marshall *et al.* (1952), except that lactic and succinic acids could not be separated under any circumstances. These two acids ran together, both as individual acids on separate columns and on paper chromatograms. By slowing the rate of increase of polarity of the solvent, the two acids were partially separated, but only very slightly. The small rise in titre occurring between acetic and fumaric acids shown in Fig. 2 may be due to lactyl-lactic acid, which

always accompanies lactic acid. When lactic acid was omitted from the acid mixture, no such rise in titre occurred, and, conversely, if lactic acid alone was run, two peaks were obtained. The larger peak was identified as lactic acid; the smaller corresponds to the slight peak found between acetic and fumaric acids.

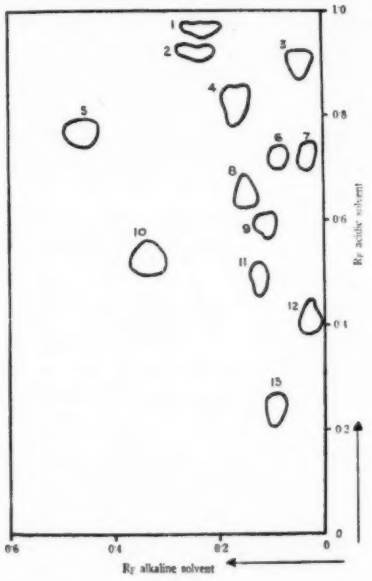

Fig. 3.

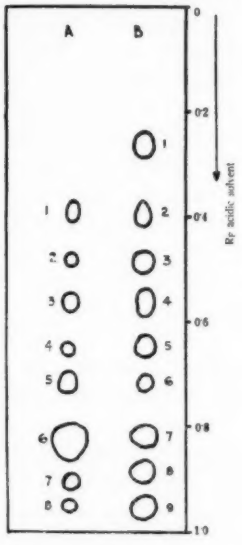

Fig. 4.

Fig. 3. Two-dimensional chromatogram of a standard acid mixture. 1, fumaric acid; 2, glutaric acid; 3, *cis*-aconitic acid; 4, succinic acid; 5, lactic acid; 6, malonic acid; 7, tricarballylic acid; 8, α-ketoglutaric acid; 9, maleic acid; 10, glycollic acid; 11, L-malic acid; 12, citric acid; 13, tartaric acid.

Fig. 4. One-dimensional paper chromatogram of A, yeast extract (≡100 mg. wet weight yeast); B, standard acid mixture.

Acids in A: 1, citric acid; 2, malic acid; 3, unknown acid running as a monocarboxylic acid in the alkaline solvent; 4, α-ketoglutaric acid; 5, unknown acid running as a dicarboxylic acid in the alkaline solvent; 6, succinic acid; 7, very faint spot given by peaks 1 and 2 in Fig. 2; 8, fumaric acid.

Acids in B: 1, tartaric acid; 2, citric acid; 3, L-malic acid; 4, oxaloacetic acid; 5, α-ketoglutaric acid; 6, tricarballylic acid; 7, succinic acid; 8, *cis*-aconitic acid; 9, fumaric acid.

Oxaloacetic acid was found to run together with *cis*-aconitic acid on the column, whereas Marshall *et al.* (1952) obtained good separation of oxaloacetic from the other acids run. Both these differences can probably be attributed to the different properties of individual batches of silica gel.

The extraction of organic acids from yeast extract in Soxhlets is far less time consuming than in liquid-liquid extractors (Umbreit, Burris and Stauffer,

1951). The method was proved quantitative in two ways: (a) known amounts of standard acids were extracted from aqueous solution and run on a silica gel column, the recoveries being estimated by titration of the individual acids; (b) known amounts of succinic acid and L-malic acid were estimated manometrically after extraction, using succinoxidase (Krebs, 1937) and a suspension of *Lactobacillus arabinosus* (Nossal, 1951) respectively.

Although the method of preparing and running the column is carefully standardized so that the fraction giving the highest titration figure for any particular acid peak rarely deviates from the average by more than five tubes, the initial pressure required to achieve a flow of 22 drops/minute varied considerably (4-7 lb./sq. inch). To maintain the rate over the whole run, the pressure was gradually increased to 9-13 lb./sq. inch.

The two peaks occurring between fractions 15-20 and fractions 35-45 in Fig. 1 (Nos. 1 and 2 respectively) could be due to the same acid in the yeast. Marvel and Rands (1950) showed that an acid, which is only slightly soluble in water, highly soluble in alcohol and insoluble in chloroform, may give two peaks. We have found with fumaric acid that when the amount placed on the column was increased, the normal peak between fractions 55-65 and a second peak between fractions 15-20 were obtained. Both of these gave two-dimensional paper chromatograms identical with that of fumaric acid. But neither of the first two peaks in yeast gave an acid spot, or at the best a very faint spot moving just faster than *cis*-aconitic acid, on paper chromatograms, although applied in theoretically sufficient quantities (Fig. 4A, No. 7). Neither peak seems to be a volatile acid which is removed with the swamping acid during the drying of the papers, since the peaks themselves and a whole aqueous yeast extract contained no volatile acid (Bartley, 1953). When the neutralized column fractions of these two yeast peaks were pooled, evaporated to dryness and re-applied to a new column instead of a paper chromatogram, only a 20 p.c. recovery was obtained. The sodium salt of the yeast acid is therefore unstable to mild heating in aqueous solution, and the failure to identify it by paper chromatography is due to its decomposition during the evaporation of the pooled column fractions.

Different batches of yeast vary markedly in the absolute amounts of the various acids present, particularly succinic acid. It has become obvious that identification by comparing the number of fractions required to elute these acids and suspected known acids from the column, or even co-chromatography with known acids, is by no means infallible. The unknown acid and the suspected acid should give identical spots on a two-dimensional paper chromatogram, using solvents giving widely different R_F values for the particular acids, and, if possible, this evidence should be supplemented with chemical and physical data.

SUMMARY.

Nine organic acids of biological interest have been completely separated on a silica gel column, making use of a solvent which automatically and gradually increases in polarity. The positions of eight other acids have been established.

Except for the keto acids, the acids are recovered to an extent of 95-102 p.c.

The maximal amount of each acid which can be applied to the column and yet completely separated varies with the position at which the acid is eluted. For those acids eluted well apart from any other, up to 30 μ equiv. have been added and quantitatively recovered. For those acids which are eluted close together, no more than 5 μ equiv. of each could be added for complete separation.

The acids are identified by two-dimensional paper chromatography. These techniques have been applied to a study of the non-volatile organic acids of yeast. Eight acids occur, of which five are fumaric, succinic, α-ketoglutaric, malic and citric acids. The other three acids remain unknown.

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INVESTIGATIONS ON GLUCOSE CATALYSIS IN BACTERIAL METABOLISM

1. PYRUVATE METABOLISM IN *LACTOBACILLUS ARABINOSUS 17–5*

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Many investigators have observed an unexplained stimulation of various enzymes in bacteria by glucose. Instances are formic hydrogenlyase in *E. coli* (Lascelles, 1948), aspartase in *Proteus vulgaris OX-19* (Trudinger, 1951), decarboxylation of malate and pyruvate by malate-adapted cells of *Lactobacillus arabinosus* (Nossal, 1951 and 1952) and a similar pyruvate system in *Lactobacillus plantarum* (Rowatt, 1951). Also, Broquist and Kohler (1953) reported that the stimulation of formic hydrogenlyase in *A. aerogenes* by yeast and liver extracts was due to the content of reducing sugars in the extracts. Forbes and Sevag (1951) found that glucose influenced the oxido-reductive proline-glutamic acid relationship in *Micrococcus pyogenes*. The present paper is concerned with the glucose stimulation (Nossal, 1952) of the pyruvate system in *Lactobacillus arabinosus 17-5*: 2 pyruvate $\rightarrow$acetoin$+2CO_2$.

EXPERIMENTAL.

Unless otherwise stated, cells used in these experiments were grown in a Pronutrin-glucose medium (Nossal, 1952), without addition of malate, at 30° C. for 17 hours, harvested by centrifugation, washed twice in distilled water, suspended in 0·1 M KCl to give approximately 20 mg. dry weight cells per ml. and stored at 0-2° C. Decarboxylation of pyruvate and acetolactate was followed with standard Warburg equipment at 30° C. in 0·05 M citrate-phosphate buffer with optimal concentrations of Mn^{++} and K^{+} (Nossal, 1952). Unless otherwise stated, air was used as the gas phase.

Glucose was estimated by the method of Folin and Malmross (1929), pentose according to Mejbaum (1939) and lactic acid according to Barker and Summerson (1941). Commercial pyruvic acid was twice distilled *in vacuo*, immediately diluted to a molar solution with distilled water and stored at 0° C. Yeast carboxylase was used to estimate pyruvate according to Krebs and Johnson (1937). Acetolactic acid was prepared by the hydrolysis of ethyl-α-methyl-α-acetoxy-acetoacetate (Krampitz, 1948). 3-Phosphoglyceraldehyde was prepared by the method of Meyerhof (1938).

RESULTS.

Decarboxylation of pyruvate. According to Nossal (1952), ageing of the cells leads to an inability to metabolise pyruvate in the absence of added glucose. By storing the cell suspension in 0·1 M KCl at 0–2° C., a consistent

ageing effect was again found, both with the basic and glucose-stimulated systems. (Basic activity is defined as the rate of CO_2 evolution in a system containing cells, 10^{-2} M pyruvate, 10^{-2} M KCl, 10^{-2} M $MnCl_2$ and buffer in a final volume of 3·0 ml. The glucose-stimulated system consisted of the basic system $+10^{-3}$ M glucose.) In older, inactivated cells, CO_2 evolution and acetoin formation could be partly restored by prolonged incubation with glucose. For the first few days, added glucose maintained a high level of pyruvate activity, whereas without glucose activity declined sharply (Table 1).

TABLE 1.

Ageing effect with and without glucose.

Warburg cups contained in the basic system 4 mg. dry wt. of cells, 10^{-2} M Na pyruvate, pH 3·8; 10^{-2} M $MnCl_2$; 10^{-2} M KCl; 0·05 M citrate-phosphate buffer, pH 3·8; final volume 3·0 c.c. Figures represent μl. CO_2/20 minutes.

Storage period (days)	0	1	2	3	5	7
Basic system	107	57	30	0	0	0
$+10^{-3}$ glucose	320	370	240	180	70	0

The Pronutrin medium did not support maximal growth of *Lb. arabinosus*. Addition to it of 10 ml./l. of the synthetic medium described by Pollack and Lindner (1942) increased the yield of cells 2-3 fold to approximately 1,200-1,500 mg. cells dry weight per litre. Cells harvested from this "enriched" medium showed a somewhat different ageing and stimulation pattern. Basic activity declined more linearly and the percentage stimulation by added glucose remained constant. This difference is seen in Fig. 1a. A quantitative study of the ageing process was carried out with both types of cells; both the rate of glycolysis and the stimulation of pyruvate decarboxylation were followed over a five-day period. The pattern and extent of glucose stimulation directly paralleled glucose utilisation (Figs. 1a and 1b). With cells grown in the basic Pronutrin medium, both the glycolytic rate and stimulation were greater after 24 hours' storage. This did not occur with cells grown in the enriched medium. The latter cells showed little decrease in their glycolytic activity over a five-day period, in contrast to "non-enriched" cells. This difference is clearly reflected in the stimulation patterns for each type: a constant glycolytic rate resulted in constant stimulation of pyruvate decarboxylation over the ageing period.

Glucose was converted quantitatively to lactic acid in all cases.

In very few cases were batches of cells obtained in which a significant oxygen uptake accompanied CO_2 evolution, whether the cells were grown under vigorously aerobic or comparatively anaerobic conditions.

The effect of inhibitors on activity and stimulation. Fluoride, iodoacetate and other known inhibitors of classical glycolysis were tested for their ability to inhibit the glucose effect. As in *Lb. plantarum* (Rowatt, 1951), 10^{-3} M fluoride inhibited the basic activity, inhibition being greater in the presence of glucose.

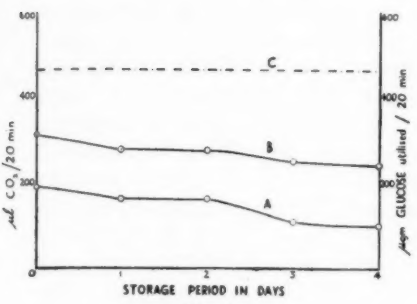

Fig. 1a.

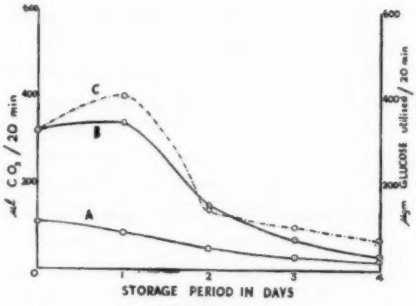

Fig. 1b.

Fig. 1a. Ageing pattern of basic and stimulated activities in "enriched" cells. Cells were harvested from the enriched medium and stored in M/10 KCl at 2° C. 5·3 mg. cells dry wt.

Curve A: basic activity; Curve B: activity with addition of 540 μg. glucose; Curve C: rate of glucose utilization.

Fig. 1b. Ageing pattern of basic and stimulated activity in normally grown cells. Cells harvested from normal Pronutrin medium and stored in M/10 KCl at 2° C. 3·9 mg. cells dry wt.

Curve A: basic activity; Curve B: activity with addition of 540 μg. glucose; Curve C: rate of glucose utilization.

Fluoride completely abolished glucose stimulation. 10^{-3} M phloridzin, cyanide and quinine had little effect on basic and stimulated systems. 10^{-3} M iodoacetate caused slight inhibition of pyruvate decarboxylation, inhibition increasing in the presence of glucose (Table 2). Addition, at serial time intervals, of iodoacetate to cells decarboxylating pyruvate in the presence of glucose immediately reduced the rate of CO_2 evolution from that of the stimulated system to that of the basic inhibited system.

TABLE 2.

Effect of inhibitors on activity and stimulation.

Basic pyruvate system as in Table 1. Glucose added 10^{-3} M. Additional compounds added to final concentration of 10^{-3} M. 4·0 mg. cells dry wt. Figures are μl. CO_2/20 minutes.

Basic	+Glucose	+Iodoacetate	Glucose +iodoacetate	+Fluoride	Glucose +fluoride
145	261	120	93	55	33

The effect of glycolytic intermediates. Decarboxylation of pyruvate by freshly harvested cells was studied in the presence of 0·01 M glucose-1-phosphate, fructose-6-phosphate, fructose-1-6-diphosphate, 3-phosphoglyceraldehyde and 3-phosphoglycerate. None had any effect on CO_2 or acetoin formation, nor were they metabolized, i.e., no lactate could be detected after incubation with the cells. Impermeability of the cell wall, or inability of the esters to enter the

glycolytic mechanism, are considered possible explanations of the inactivity of these compounds.

Adaptation experiments. *Lb. arabinosus* can be adapted to use other sugars besides glucose as primary energy substrate by normal serial subculture. Such cells metabolized pyruvate exactly as glucose-grown cells, but stimulation of pyruvate activity was maximal with the sugar to which the cells had been adapted. With glucose-grown cells, galactose had little or no stimulatory effect on pyruvate decarboxylation. When galactose was used for growth of a galactose-adapted strain, results as in Table 3 were obtained. Galactose utilization and lactate balance experiments were carried out with galactose-adapted cells. Galactose stimulation was found to parallel its utilization as in the case of glucose. One molecule of galactose yielded two of lactic acid. Also, where a second sugar or derivative is known to be an intermediate in the metabolism of the adaptive sugar used, maximal stimulation was produced by these intermediates as well, whereas in the unadapted cells all were inactive. For example, pyruvate decarboxylation by lactose-grown cells was stimulated by lactose, galactose and glucose, the former two sugars being inactive in unadapted cells (Table 3). Attempts to adapt the cells to gluconate were unsuccessful.

TABLE 3.

Comparative stimulations of glucose-, galactose- and lactose-grown cells.

Warburg cups contained basic system as in Table 1. Figures represent μl. CO_2/20 minutes. All cells freshly harvested.

Exp.	Mg. dry wt. cells	Cultivation	Basic	$+10^{-3}$ Galactose	$+10^{-3}$ Glucose	$+10^{-3}$ Lactose
1a	2	Glucose cells	36	40	190	—
1b	3	Galactose „	51	121	190	—
2a	2·3	Glucose cells	46	47	153	—
2b	2·1	Galactose „	64	130	160	—
3a	2·2	Glucose cells	28	32	147	36
3b	3·1	Lactose „	49	150	160	170

Experiments with pentose-adapted cells. Pentoses (as sole energy source) are unable to support growth of the normal laboratory strain of *Lb. arabinosus* previously maintained on glucose media, and do not stimulate pyruvate decarboxylation in hexose-grown cells. Serial subculture in 1 p.c. glucose+1 p.c. DL-arabinose and finally in 2 p.c. DL-arabinose media produced a strain capable of growing on arabinose as the sole carbon source. Suspensions of cells grown on DL-arabinose were tested for pyruvate decarboxylation, pentose stimulation and metabolism. Such cells decarboxylated pyruvate like cells grown on glucose, but stimulation of activity was also produced by D-arabinose, L-arabinose, D-ribose, adenosine and adenylic acids (Table 4). Therefore, cells adapted to use arabinose were simultaneously adapted to use ribose in agreement with observa-

tions of Lampen and Peterjohn (1951). Arabinose and adenosine followed a similar pattern of rate of utilization to that of glucose, both compounds yielding one molecule of lactic acid. Trudinger (1953) also obtained evidence that stimulation of aspartic acid deamination by adenosine in *Proteus vulgaris* is due to utilization of the pentose portion of the molecule.

TABLE 4.

Effect of pentoses and derivatives on pyruvate decarboxylation in DL-arabinose-grown cells.

Basic system as in Table 1. Additional substrates added to concentration 10^{-3} M. Figures are μl. CO_2/20 minutes. 5·2 mg. dry wt. cells.

Basic	Glucose	L-arabinose	D-arabinose	D-ribose	Adenosine	Adenosine-3-phosphate	Adenosine-5-phosphate	Adenine
70	204	208	171	183	203	230	230	97

The effect of substrate concentration on glucose stimulation. The rate of pyruvate decarboxylation in the absence of concurrent glycolysis increased linearly to a final concentration of 2×10^{-2} M pyruvate, further increases in concentration to 5×10^{-2} M having little effect. In the presence of glucose, the curve followed an elevated parallel pattern with a constant stimulation ratio (Fig. 2). Little variation in pattern was obtained in high sodium or high potassium systems (cf. Trudinger, 1953).

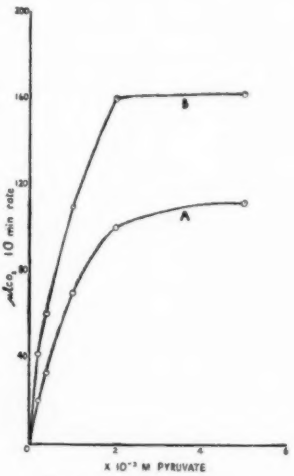

Fig. 2. Substrate concentration curve in basic and stimulated systems. 4·3 mg. cells dry wt. Curve A: basic system; Curve B: activity with addition of 540 μg. glucose.

The effect of prolonged pre-incubation with glucose on stimulation. Experiments were carried out to determine the effect of pre-incubating fresh cells with glucose for varying periods before adding pyruvate. Glucose utilization and lactate formation were determined simultaneously. Cells were pre-incubated with 10^{-3} M glucose (=540 μg./3 ml.) in Warburg cups for intervals of 20, 40, 60 and 90 minutes before pyruvate was tipped in. Control cups, without glucose, were included to measure enzyme stability. Usually, the basic pyruvate activity of the cells was only slightly affected by prolonged pre-incubation without substrate. In zero time controls, in which pyruvate decarboxylation commenced at the same time as glycolysis, the usual large immediate stimulation of pyruvate decarboxylation was found. After 60 minutes' incubation, less than 20 μg. of the added glucose remained, and after 90 minutes, none. When pyruvate was tipped

TABLE 5.

Effect of prolonged preincubation with glucose on stimulation produced.

Cups contained: Na pyruvate, 10^{-2} M; glucose, 10^{-3} M; basic system as in Table 1; 4·2 mg. cells dry wt.
Figures represent μl. CO_2 evolved in time interval indicated.

Interval from tipping pyruvate (minutes) ↓	Preincubation times → Zero		20 minutes		40 minutes		60 minutes		90 minutes	
	Basic system	+Glucose	Basic system	+Glucose	Basic system	+Glucose	Basic system	+Glucose	Basic system	+Glucose
10	66	132	68	180	64	118	77	81	58	65
20	171	330	171	270	160	220	188	194	143	148
30	286	455	278	330	250	330	257	290	222	228
40	377	510	360	420	330	390	350	386	320	320

in at 90 minutes, the rates of CO_2 evolution from the basic and glucose incubated systems were identical (Table 5). After a second addition of 540 μg. glucose at this stage, immediate stimulation was again produced, showing that the cells were not inactivated by previous incubation with glucose. This is good evidence that stimulation is produced only during active glycolysis. The stimulatory process seemingly involves a coupling at some point between glycolysis and the pyruvate system. Results of this type do not favour the hypothesis of cofactor generation by glucose.

Decarboxylation of acetolactate. Juni (1952) and other workers have shown that extracts of various organisms, including *Aerobacter aerogenes, Staphylococcus aureus* and *Streptococcus faecalis* can decarboxylate acetolactate to acetoin and CO_2. Juni fractionated extracts of *Aerobacter aerogenes* and obtained two fractions, one forming acetolactate from pyruvate, the second decarboxylating acetolactate to acetoin and not affecting pyruvate. Whole suspensions of *Lb. arabinosus 17-5* enzymatically decarboxylated acetolactate at rates indicating that this compound could be an intermediate in acetoin formation. The decarboxylation was stimulated by Mn^{++} and proceeded optimally at pH 3·8. Activity was low at pH 6·0. In contrast to pyruvate decarboxylation, evolution of CO_2 from acetolactate was only very slightly accelerated by concurrent glycolysis (Table 6).

TABLE 6.

Decarboxylation of acetolactate.

Basic pyruvate system as in Table 1. Basic acetolactate system as in Table 1, but without $MnCl_2$ and with 0·6 ml. M/10 DL-acetolactate added. Glucose added 10^{-3} M, $MnCl_2$ added 10^{-3} M. Exp. 1: 4 mg. dry wt. cells; exp. 2: 4·2 mg. dry wt. cells. Figures are μl. CO_2/20 minutes.

pH→	3·8	3·8	3·8	3·8	3·8	5·1	6·0
Exp.	Basic pyruvate	Pyruvate +glucose	Basic aceto-lactate	Basic aceto-lactate +Mn^{++}	Aceto-lactate +Mn^{++} +glucose	Aceto-lactate +Mn^{++}	Aceto-lactate +Mn^{++}
1	90	270	120	170	180	—	—
2	140	230	100	120	128	50	14

DISCUSSION.

A dual problem exists in the study of glucose catalysis of the pyruvate system in whole cells of *Lb. arabinosus*. Both the exact mechanism of pyruvate decarboxylation and acetoin formation and the mechanism of glucose stimulation are unknown. The present experiments show that the glucose effect may be called the concurrent glycolysis effect. Glucose is usually considered to be converted to lactate by classical anaerobic phosphorylative glycolysis in this organism, and evidence is now obtained that pentoses also follow standard path-

ways. Any sugar or derivative which is metabolized by the cells produces stimulation of pyruvate decarboxylation so consistently that sugar utilization may be detected by this effect. Since the decarboxylation of pyruvate in cells adapted to arabinose is stimulated by arabinose, and also by ribose, adenosine and its derivatives, it appears that arabinose is metabolized through compounds with ribose configuration. This is in agreement with the work of Lampen and Peterjohn (1951) on the specificity of pentose metabolism in *Lb. pentosus*. These authors concluded that xylose and arabinose were converted to ribose derivatives before cleavage of the pentose chain occurred. According to the studies of Horecker and Smyrniotis (1951), this intermediate is probably ribulose-5-phosphate.

In acetoin forming systems, Ochoa (1951) postulated that pyruvate gives rise to CO_2 and an acetaldehyde-enzyme complex which may either condense with pyruvate to give acetolactate or with aldehydes to give acyloins. Juni's (1952) work leaves little doubt that acetolactate is a true intermediate in *A. aerogenes*. On the basis of kinetic data and the effects of acetate, Happold and Spencer (1952), again with *A. aerogenes*, put forward a scheme in which the aldehyde-enzyme complex condensed with acetate to give diacetyl and then acetoin by reduction, relegating acetolactate to an alternative pathway. These workers considered the possibility of acetate condensing with lactate, involving oxidation and reduction of pyruvate, and stated that the known effects of hydrogen donors may be accounted for in such a reaction. Using purified pyruvic carboxylase from wheat germ, Singer and Pensky (1951) showed that pyruvate decarboxylation and acetoin formation are catalysed by a single enzyme. Schweet *et al.* (1952) obtained evidence to indicate that acetoin is produced during pyruvate oxidation by the pyruvic oxidase of pigeon muscle, a C_2 fragment being formed which could be oxidized to acetate or condensed with a similar fragment to give acetoin. Hydrogen donor function is also indicated in such a reaction. In *Lb. arabinosus*, preliminary experiments with cell-free extracts seem to indicate a similar pathway.

The precise integration of glycolysis with mechanisms such as these cannot be visualized at present. It is also questionable whether there is a specific focal point of the action of glucose in this system, and perhaps a similar or common focal point in all the glucose-stimulated systems listed above. Generation by glycolysis of a coenzyme of the pyruvate system in *Lb. arabinosus* is not a likely explanation of the glucose effect in view of the stimulation patterns obtained. Maintenance of a coenzyme or enzyme in a reduced state must be considered a possibility in the light of these results and of current hypotheses on the function of hydrogen donors in acetoin systems. Maintenance or influx of cofactor metal ions does not seem to be the explanation, as the system requires only Mn^{++}. The potassium effects observed by Nossal (1952) were considered supplementary to glycolysis. Cell-free preparations, also, have now been shown to have no

potassium requirements. The fact that all the systems stimulated by glucose so far reported are concerned with the cellular metabolism of an acid radical may lead to the idea of active transport being the answer. However, results obtained here with acetolactate, and with pyruvate concentration curves, do not favour this hypothesis.

SUMMARY.

The stimulation of pyruvate decarboxylation and acetoin formation in *Lactobacillus arabinosus 17-5* by glucose occurs only during concurrent glycolysis of the added glucose.

Over an ageing period of five days, the pattern of stimulation directly parallels the pattern of glycolysis.

Stimulation is produced by any sugar to which the cells are adapted.

Cells adapted to arabinose are stimulated by, and can utilize, ribose and derivatives, suggesting that arabinose is metabolized through intermediates with ribose configuration.

Whole cell suspensions decarboxylate acetolactic acid at rates equivalent to those of pyruvate, the decarboxylation being stimulated by manganese, but not by concurrent glycolysis.

Various theories of the mechanism of glucose catalysis are discussed.

Acknowledgments. Thanks are due to Mr. P. A. Trudinger for helpful discussions, and to Dr. H. J. Rodda, of the Department of Chemistry, for assistance in the preparation of acetolactate.

Note added in proof: After this paper had been submitted, Moat and Lichstein (1953) reported that in vacuum dried cells of *Lactobacillus arabinosus*, adenosine triphosphate (ATP) could replace glucose as a catalyst for production of acetoin and CO_2 from pyruvate. No data were given for the effect of ATP in fresh cells. The glucose effect disappeared in vacuum dried cells on ageing. These facts were taken as evidence for a permeability-energy phenomenon to explain the glucose effect.

In this laboratory, no stimulation by ATP was observed in vacuum dried cells. Stimulation by ATP and other adenosine derivatives was readily demonstrable in untreated pentose adapted cells (see above), and with these, stimulation was due to metabolism of the pentose moiety. Glucose utilization and pyruvate stimulation were obtained in vacuum dried cells after 24 hours ageing. Further ageing resulted in loss of stimulation but also in failure to utilize glucose. As we have shown that stimulation of the pyruvate system occurs only during concurrent glycolysis, this disappearance of glucose stimulation is thus not necessarily due to the removal of a permeability barrier.

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STUDIES ON THE HEPATO-ENCEPHALOMYELITIS VIRUS (HEV)

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The paper recording the isolation of the hepato-encephalomyelitis virus (HEV) described many of the properties of the infective agent in the early passages after its isolation and identification in suckling mice (Stanley *et al.*, 1953a). Further passages showed that the agent possessed an affinity for central nervous system tissue which resulted in a gradually changing clinical picture in infected mice.

The virus was isolated from the faeces of an aboriginal child who some months later developed alopecia and conjunctivitis—two symptoms associated with infection of infant mice. As originally isolated, the HEV produced a wide variety of signs in suckling mice, viz., jaundice, oily hair effect, paralysis, tremors, inco-ordination, retardation of growth, conjunctivitis and alopecia. Most of the animals infected died. Complement-fixation tests with a purified infected suckling-mice brain antigen revealed antibodies in the patient's serum eleven months after isolation of the agent and in the pooled serum of aboriginal natives from the Northern Territory, but not in the serum of any white person tested. The growth of the virus on the chorio-allantois of developing chick embryos was demonstrated. The possibility of a mixture of infective agents was considered in view of the recent demonstration of Gledhill *et al.* (1952) that a mouse hepatitis virus could be resolved into two filterable components. The evidence so far produced indicates the homogeneity of the HEV. A preliminary examination revealed that the virus was unusually resistant to heat and germicides. Experiments to determine the size of the virus were not reported.

Arising from these preliminary observations were several problems that required further study before any great extension of the work could be undertaken. The major difficulties lay in the properties of the virus itself with respect to the variations produced by laboratory manipulation. Other problems were concerned with the failure to develop a satisfactory test for neutralizing antibodies, the presence of complement-fixing antibodies in the sera of aboriginal natives, the physical properties of the virus, and the unusual array of signs produced in suckling mice and whether or not these were the result of a mixed infection.

Further investigations have therefore been carried out and are presented in the following sections: I, Variation; II, Physical properties; III, Neutralization tests; IV, Complement-fixation tests; V, Discussion on the nature of HEV.

I. VARIATIONS OF THE HEPATO-ENCEPHALOMYELITIS VIRUS.

Originally it was found possible to divide the course of infection in suckling mice into five phases, viz., i, primary liver invasion; ii, encephalitis and oily hair effect; iii, quiescence; iv, alopecia; v, secondary jaundice.

Continuous suckling-mouse passage of infected brains soon resulted in our inability to demonstrate these five phases as the virus appeared to become consistently neurotropic. This section describes in greater detail this variation of the HEV and to what extent it may be controlled by laboratory manipulation.

EXPERIMENTAL.

Description of mice infected with variants.

The variants may be easily recognized by studying the appearance of infected suckling mice. The virus as it was first isolated and passaged will be referred to as H.HEV, while the variant arising as a result of laboratory manipulation will be designated *N*.HEV.

H.HEV produces in mice the characteristic oily hair effect (OHE) and jaundice after an incubation period of nine to twelve days. At this time the mice may also show signs of C.N.S. involvement, manifested by inco-ordination and tremors. The few mice surviving follow the pattern outlined in an earlier publication and *may* pass through phase 3 (quiescence), phase 4 (alopecia) and phase 5 (secondary jaundice). The appearance of mice infected with H.HEV is shown in Figs. 1 and 2 of our earlier paper (Stanley *et al.*, 1953a).

Mice infected with *N*.HEV rapidly show evidence of C.N.S. involvement five days after inoculation by the intraperitoneal route when they show inco-ordination, tremors and/or paralysis, and die a few hours after onset of symptoms. They do not show OHE, jaundice or alopecia. From the appearance of infected suckling mice it was thought that the H.HEV was primarily hepato-tropic and the *N*.HEV primarily neurotropic. Whether this assumption is justified or not, lesions in the brains, spinal cords and livers were observed with both.

Maintenance of the variants.

It was soon found that with passage of H.HEV infected mouse tissue by the intraperitoneal route in suckling mice the OHE and jaundice became harder to demonstrate. After eight passages these effects were no longer demonstrable and the mice died within four or five days showing signs of C.N.S. involvement. A similar change was brought about by passage on the chorio-allantois of 11-day-

old chick embryos. The change, H.HEV→*N*.HEV, appears to be irreversible *once complete,* and may take place more rapidly when the agent is placed under apparently adverse conditions.

As it was found that infant mice were susceptible to infection when given virus by the intracerebral, intraperitoneal, intranasal or intradermal routes, it was decided to use different routes of inoculation in, and different infected organs of, suckling mice in order to minimize or prevent the variation.

It was observed on one occasion that a complement-fixing antigen (containing infective virus), when given by the oral route to suckling mice, caused death which followed the signs of H.HEV infection after ten days, although this same preparation when given to suckling mice by the intra-peritoneal route caused the mice to die following the classical signs of *N*.HEV infection after five days. The possibility of maintaining the H.HEV variant in its original form by passages of infected tissues by the oral route was thereby suggested. Accordingly, ten successive passages *per os* of livers infected with H.HEV were carried out. An identical experiment was performed with *N*.HEV. The results revealed that the H.HEV variant showed far less tendency to vary to *N*.HEV than when passaged by the intraperitoneal route. Ten successive passages *per os* of *N*.HEV infected livers, on the other hand, showed no tendency to revert to H.HEV. The differing incubation periods of the two variants (5 days for *N*.HEV and 10 days for H.HEV) persisted. This process, maintaining H.HEV and preventing its variation to *N*.HEV, was extended by passaging intestinal contents of infected mice by the oral route (seven passages) and eventually led to the development of the present "contact" technique used for the maintenance of H.HEV. With the technique now adopted, a living infected mouse is placed with a litter of mice 24 hours old. Following the usual incubation time (about ten days), the suckling mice in this litter show the OHE, jaundice and incoordination, characteristic of H.HEV infection. Any of the infected mice may now be selected for transference to another litter 24 hours old. By this natural route of infection the virus has shown no tendency to vary to *N*.HEV over five passages. Mice more than three days old could not be infected by this "contact" technique.

In an attempt to reconvert *N*.HEV into H.HEV, and to maintain H.HEV in its original form, monkeys were given virus by the oral route. Three types of monkeys were used. Monkey No. 62 (*M. nemestrinus*) and monkey No. 65 (*M. irus*) were given H.HEV *per os.* Monkey No. 63 (*M. mulatta*) was given *N*.HEV *per os.* Approximately 0·5 ml. of a 10 p.c. infected tissue suspension was fed to the monkeys. Neither variant appeared to be changed by this treatment. All monkeys appeared to be symptomless. Evidence of infection *via* the alimentary tract was indicated by the excretion of virus in the stools (H.HEV only), the development of complement-fixing and neutralizing antibodies and the finding of classical liver lesions at post-mortem examination (see Table 1).

TABLE 1.

Infection of monkeys with N.HEV and H.HEV by the oral route.

Type	Inoculum	Rise in titre of C.F.A.	Rise in titre of N.A.	Virus in faeces	Liver lesions
M. nemestrinus No. 62	H.HEV *per os* 3/2/53	+	+	2 days ++++ 3-9 days −	++++
M. mulatta No. 63	*N*.HEV *per os* 19/2/53	+	+	7 days −	−
M. irus No. 65	H.HEV *per os* 11/3/53	+	+	2-3 days ++ 6-8 days −	+

C.F.A.=complement-fixing antibodies. N.A.=neutralizing antibodies.

After ten successive suckling-mouse passages *per os* of H.HEV infected livers, all mice still showed the OHE and jaundice after an incubation period of eleven days. The brains of these mice were used to ascertain how many passages were required by the intraperitoneal route for the *N*.HEV to become predominant. Of the five litters inoculated with a 10 p.c. brain suspension diluted from 10^{-1} to 10^{-5}, all mice showed a peritoneal exudate, a few mice showed jaundice at 10^{-4} and 10^{-5} dilutions, but all died before the tenth day after inoculation and the OHE was not seen. The passage of the brains of the mice from litters inoculated with 10^{-1}, 10^{-2} and 10^{-3} dilutions resulted in mice developing the *N*.HEV syndrome after an incubation period of five days. Nevertheless, after seven passages of infected suckling-mouse brain by the intraperitoneal route, the H.HEV syndrome could be demonstrated by inoculation of livers by the oral route. This indicated that the H→*N* change was not at this stage complete.

Often the first step in the H→*N* change appeared to be a blood-stained peritoneal exudate. From mice showing this symptom both variants could usually be recovered. When the H→*N* change is complete, mice rarely show a peritoneal exudate.

Fat estimations of intestinal contents.

Infections with H.HEV were invariably characterized by the prolific production of faecal material of lemon yellow colour. This occurred at the time of onset of the OHE, which may result from the smearing of faecal material on the skin, especially as the OHE appears first in the anal region. The production of faeces by H.HEV-infected mice is many times greater than that of normal mice of the same age. The total fat of the intestinal contents of infected mice,

and of normal mice of the same age, was estimated and the results are recorded in Table 2.

Age susceptibility of mice.

Mice older than three days did not show evidence of infection with H.HEV when inoculated by the intraperitoneal route. The *N*.HEV variant was shown to infect mice a few days older, and the results of an experiment illustrating this susceptibility are presented in Table 3.

TABLE 2.

Fat estimations of normal and infected mouse intestinal contents.

Material	P.c. fat
Normal intestinal contents	4·6
HEV intestinal contents	12·9
HEV faeces	29·0
Normal faeces	not collected

TABLE 3.

Age susceptibility of mice given HEV by the intraperitoneal route.

	Age of litter in days			
Variant	3	6	10	21
N.HEV.	9/9	5/5	7/9	0/5
H.HEV	8/8	0/8	0/8	0/8

Numerator=number of mice dying as a result of infection.

Denominator=number of mice in litter inoculated.

This difference between H.HEV and *N*.HEV is less important when one considers the difference in incubation periods of the two agents. The incubation period for *N*.HEV is five days (average), so that mice infected on the tenth day after birth would be fifteen days old when symptoms of infection first appear. The incubation period for H.HEV is twelve days (average), so that mice infected on the third day after birth would also be fifteen days old when symptoms first appear.

When *N*.HEV (in high concentration) was given by the intracerebral route to adult mice, infection was difficult to demonstrate. Only a few of the mice showed inco-ordination followed by death. Presence of the virus in the brain was demonstrated by inoculation of suckling mice and by the appearance of haemorrhages, perivascular cuffing and necrosis in sections. The virus could not be transmitted by further passage in adult mice, although repeated attempts were made to do this. Intracerebral inoculation of adult mice with H.HEV produced no demonstrable evidence of infection.

Distribution of virus in the tissues.

In studying the pathogenesis of infection with H.HEV it was shown that there was a primary invasion of the liver, followed by an invasion of the C.N.S. and the concentrations of virus in the liver, brain, lung, peritoneal-exudate and intestinal contents were determined (Stanley *et al.*, 1953a). It seemed likely that a viraemia took place, but blood was not collected for testing during these

early experiments. Furthermore, it was not known whether a viraemia occurred in *N*.HEV-infected mice. In the following experiment four litters of eight mice were inoculated with *N*.HEV by the intraperitoneal route. At 24-hour intervals thereafter four mice were bled and the pooled blood titrated for virus by the intraperitoneal route. The results are recorded in Table 4. At 156 hours, the mice whose blood contained virus at an ID_{50} of 10^{-4} also showed virus in their pooled brains and livers to a titre of 10^{-5}.

TABLE 4.

ID_{50} of blood collected after inoculation of N.HEV by the intraperitoneal route into suckling mice.

	Time in hours after inoculation						
	24	48	72	96	108	132	156
ID_{50}	10^{-1}	10^{-4}	$>10^{-3*}$	$10^{-4.8}$	$>10^{-3*}$	$>10^{-3*}$	10^{-4}

*These titrations were not carried out further than 10^{-3}.

Growth on the chorio-allantois of chick embryos.

In our previous communication on HEV it was shown that pocks were produced on the chorio-allantois following the inoculation of 11-day chick embryos with HEV-infected mouse brain or liver. On checking these results it was found that the preparations used probably consisted of both H and *N* variants. On continued passage, the number of foci produced decreased progressively and none was detectable by the fourth passage. This work was repeated using separate suspensions of H.HEV and *N*.HEV. The H.HEV preparation came from the infected livers of "contact" mice Passage 1, and the *N*.HEV from the stock neurotropic variant suspensions which had been used for the other tests reported in this paper. The details of this experiment are recorded in Table 5.

The pocks appeared to be identical with each variant. A description of stained sections of chorio-allantoic membranes showing pocks is given in the earlier publication (Stanley *et al.*, 1953a). One difference was observed between *N*.HEV and H.HEV following successive passages of infected membranes on the chorio-allantois: H.HEV could not be recovered (by suckling-mouse inoculation) in the absence of pocks, whereas *N*.HEV could be recovered from membranes which showed no macroscopic abnormality. These results confirm our earlier observations.

SUMMARY.

A description of the neurotropic variant (*N*.HEV) and the hepato-tropic form (H.HEV) of the hepato-encephalomyelitis virus is given. It was shown that H.HEV could be maintained in its original form, either by continuous passage of infected tissue by the oral route in suckling mice, or by the "contact"

technique which simulates a natural route of infection. The rapidity of change to neurotropism and the correspondingly shortened incubation period constituted a major difficulty in studying the H.HEV, and the conditions necessary to produce this change were examined. Once the change to neurotropism is completed it is thought to be irreversible. A viraemia was demonstrated shortly after infection of mice with *N*.HEV by the intraperitoneal route. Estimations revealed a large increase in the fat content of faecal material of H.HEV-infected mice.

TABLE 5.

Behaviour of HEV during five successive passages on the chorio-allantois of 11-day-old chick embryos.

Variant	Passage number	Appearance of C.A. membrane 48 hours after inoculation	Incubation period (days) in suckling mice following inoculation of C.A. membranes by I.P. route	Incubation period (days) in suckling mice following inoculation of C.A. membranes by Oral route	Symptoms of infection in suckling mice
H.HEV	Primary	numerous white pocks	7	7	OHE, jaundice, peritoneal exudate, death
	I	distinct white pocks	7	8	
	II	a few pocks	5	9	
	III	very occasional pocks	5	10	
	IV	no pocks	—	—	
	V	no pocks	—	—	
N.HEV.	Primary	numerous white pocks	4	8	
	I	thickened and gelatinous, no pocks	4	8	tremors, emaciation, peritoneal exudate, death
	II	thickened and gelatinous, no pocks	6	11–12	
	III	thickened and gelatinous, no pocks	7	9–11	
	IV	no pocks	7	—	
	V	no pocks	6–7	—	

I.P.=intraperitoneal. C.A.=chorio-allantoic. —=mice did not become infected.

Monkeys suffered a symptomless infection when given virus by the oral route. Virus was excreted in the faeces, there was liver damage, and both complement-fixing and neutralizing antibodies were formed. These observations are discussed in another section.

II. PHYSICAL PROPERTIES OF HEV.

It was shown in our preliminary communication that HEV was resistant to the actions of penicillin, streptomycin and aureomycin. Furthermore, it could not be inactivated over a wide range of pH values (2·2 to 8·0), nor by shaking with ether. It survived heating at 56° C. for two hours and exposure to 2 p.c. lysol, 0·25 p.c. formalin and 0·25 p.c. phenol at room temperature for one hour. such markedly resistant properties warranted further investigation.

EXPERIMENTAL.

Sensitivity to heat and germicides.

These tests were carried out with a 10 p.c. suspension of *N*.HEV-infected suckling mouse brains in broth, cleared by centrifugation at 3,000 r.p.m. for ten minutes. The virus was not inactivated by exposure for one hour at room temperature to 1 p.c. H_2O_2, 1 p.c. phenol or 3 p.c. formalin. On the other hand, 70 p.c. ethanol for one hour at room temperature or 3 p.c. formalin at 56° C. for thirty minutes completely inactivated the virus.

Estimation of size of HEV.

Both H.HEV and *N*.HEV were shown to pass through bacteria tight filters (Seitz E.K. and Berkefeldt candles). For filtration tests through gradocol membranes the *N*.HEV strain was derived from eight suckling mouse-brain passages which followed ten successive passages on the chorio-allantois of developing chick embryos. This variant produced a very consistent picture following inoculation of suckling mice, and the H→*N* change appeared to be irreversible. The H.HEV strain was derived from infected suckling-mouse livers following ten passages of infected liver by the oral route. A 2 p.c. suspension of infected suckling-mouse brains in ¼ strength broth was used for *N*.HEV and a similar suspension of infected intestinal contents for H.HEV. It was found that both preparations were identical in their behaviour. The virus readily passed through membranes having an average pore diameter (A.P.D.) of 340 mμ, but was completely retained by membranes having an A.P.D. of 110 mμ. It is likely, therefore, that HEV has a particle size of approximately 100 to 150 mμ.

SUMMARY.

It was shown that HEV is remarkably heat resistant, surviving temperatures of 56° C. for two hours, or 60° C. for thirty minutes, as well as resisting inactivation by 3 p.c. formalin, 1 p.c. phenol, 1 p.c. H_2O_2 and some common antibiotics. At room temperature 70 p.c. ethanol was the only germicide tested which completely inactivated HEV. Filtration through gradocol membranes of known pore size revealed that both variants behaved identically and probably consist of particles between 100 and 150 mμ in diameter.

III. NEUTRALIZATION TESTS WITH THE HEV.

It was demonstrated earlier that neutralization tests with HEV could not be performed as successfully as complement-fixation tests (Stanley *et al.*, 1953a). The explanations for the inability to demonstrate neutralizing antibodies to as high a titre as complement-fixing antibodies were not forthcoming at this stage, and the results were in contrast with our observations with similar tests performed with the Coxsackie group of viruses (Stanley *et al.*, 1953b, c). It became important to devise a neutralization test for HEV which would assist in the interpretation of our results with the complement-fixation tests. This section describes the technique of a neutralization test which is believed to be a more sensitive test for HEV antibody than the neutralization test previously described. The antibody response to infection of laboratory animals and the detection of neutralizing antibodies in human sera are reported.

Experimental.

The neutralization tests.

These were carried out in suckling mice not more than 24 hours old. It had been our custom to use the intraperitoneal route almost exclusively in our Coxsackie investigations, but, as this did not prove very satisfactory with HEV, it was decided to study variations of the standard technique.

Five variations of the neutralization test were used. In each case serial double dilutions of serum from 1/10 to 1/640 were made in broth. To each dilution was added an equal volume of stock virus suspension containing 100 ID_{50} of virus as determined by intraperitoneal inoculation. The serum-virus mixtures were allowed to stand for the times recorded in Table 6, after which they were inoculated into suckling mice. Intradermal inoculations were carried out in the skin over the occipital region of the skull. The animals were observed daily for three weeks. Survival was taken to indicate protection and 50 p.c. end-points for the serum dilutions were obtained. The variations of technique and the results obtained are recorded in Table 6.

TABLE 6.

HEV neutralizing antibodies in hyperimmune mouse serum.

Time of room-temperature incubation of serum-virus mixtures	Route of inoculation	50 p.c. protection end-point titre*
1 hour	intraperitoneal	1:54
24 hours	intraperitoneal	1:58
72 hours	intraperitoneal	1:59
1 hour	*per os*	1:37
1 hour	intradermal	1:690

* Normal mouse serum <1:10.

Because of the greater sensitivity of the intradermal neutralization test, the technique was further exploited. Before deciding on the concentration of virus to be used in the test, the ID_{50} of both *N*.HEV and H.HEV was determined by the intradermal route. The results are compared in Table 7 with those obtained by the intraperitoneal or oral routes.

TABLE 7.

Concentrations of HEV in stock suspensions prepared from infected suckling-mouse brains (N.HEV) or infected livers (H.HEV) as estimated with different routes of inoculation.

Route of Inoculation	ID_{50} *N*.HEV	H.HEV
Intradermal	10^{-7}	10^{-4}
Intraperitoneal	$10^{-8.8}$	10^{-2}–10^{-5}
per os	$10^{-4.1}$	$10^{-3.8}$

Results of neutralization tests carried out using the intradermal method.

The first tests were performed with hyperimmune mouse serum prepared against *N*.HEV after it had received ten successive passages on the chorio-allantois. H.HEV was used at a concentration of 10^{-3} and *N*.HEV at a concentration of 10^{-6}. The results are recorded in Table 8.

TABLE 8.

Intradermal neutralization tests with N.HEV hyperimmune mouse serum against H.HEV and N.HEV.

Virus	Reciprocal of dilutions				
	40	80	160	320	640
*N.*HEV	0/7	0/7	2/7	3/7	6/7
H.HEV	0/8	0/8	0/8	6/8	9/10

Numerator=number of mice dying as a result of infection.
Denominator=number of mice in litter inoculated.

TABLE 9.

Intradermal neutralization tests with human and monkey sera against N.HEV.

Serum collected from	Reciprocal of 50 p.c. end-point titre
Monkey 62 (H.HEV *per os*)	
after 16 days	10
after 27 days	15
after 34 days	>30
Monkey 63 (*N.*HEV *per os*)	
after 4 days	<10
after 7 days	5
after 14 days	25
after 21 days	25
Infective hepatitis patients (white population)	
1	<10
2	<10
3	<10
4	<10
[1]Marlowe (after 11 months)	30
[2]Katherine P.H.S. (17/10/52)	>15
[2]Bungalon 15	15
[2]Bungalon 7	15
[2]Bungalon 4	10
Korea P.H.S. (13/9/52)	<10
Philippines P.H.S. (20/10/52)	<10
[2]Bungalon 1	<10
[2]Yuendumu 22	<10
Barbaraui	<10

[1]Patient from whom virus was isolated. [2]Aboriginal sera.

These observations confirmed our earlier belief that *N.*HEV and H.HEV were antigenically homogeneous. Furthermore, although they indicated that either variant would probably be satisfactory in carrying out neutralization tests with human sera, it was felt that *N.*HEV would be more satisfactory for this purpose because of the shorter incubation period and rapid death of the animals. To determine whether or not this would prove to be the case, the reactions against *N.*HEV of a few sera from patients with infective hepatitis (white population) and from aborigines were studied. The results with these sera and with the sera of monkeys given virus by the oral route are recorded in Table 9.

SUMMARY.

An intradermal method of performing neutralization tests is described. Experiments revealed that this method is a more sensitive one for the detection of protective antibody than the neutralization tests carried out with intraperitoneal and *per os* inoculations. The results of neutralization tests with the sera of infected monkeys and from Australian aborigines are recorded, but are discussed in a later section of this paper.

IV. COMPLEMENT-FIXATION TESTS WITH THE HEV.

It was shown that the complement-fixation test appeared to be a satisfactory technique for the detection of antibody to the hepato-encephalomyelitis virus (HEV) (Stanley *et al.*, 1953a). In the same paper it was observed that complement-fixing antibody was demonstrable only in the sera of Australian aborigines and not in any of the sera examined from the white population, which included patients with infective hepatitis, homologous serum jaundice, Weil's disease, syphilis and infectious mononucleosis. It was then observed that the aboriginal sera reacting positively with HEV also gave a positive Wassermann reaction, and, in view of the likelihood of non-specific fixation of complement by suckling-mouse brain suspensions with sera giving a positive Wassermann reaction (de Boer and Cox, 1947; Espana and Hammon, 1947), it was decided to investigate the matter further. This section describes some serological tests carried out in an attempt to clarify the reaction occurring between aboriginal sera and HEV.

EXPERIMENTAL.

Preparation of complement-fixing antigens.

These were prepared from the brains or heads of infected 7- to 12-day old mice. Normal mice of the same age were used in the preparation of the control antigen. A 20 p.c. brain suspension was made in 0·9 p.c. saline in a Waring Blendor. The suspension was frozen and thawed three times, then cleared by centrifugation at 4,500 r.p.m. at 0° C. for 30 minutes. The supernatant fluid was extracted three times with twice its volume of diethyl ether. The last traces of ether were removed *in vacuo*, and the aqueous extract frozen and thawed six times. The extract was then cleared by spinning for 30 minutes at 30,000 r.p.m. in the No. 40 rotor of a Spinco ultracentrifuge. The supernatant fluid was recovered and frozen and thawed twice. The resulting precipitate was removed by centrifugation and the supernatant fluid was stored at −23° C. Such antigens were still infective and no preservative was added.

Technique of the complement-fixation test.

Volumes of 0·25 ml. were used for each reagent. The fixation of 2 M.H.D. of complement was allowed to occur at 4° C. overnight. After the addition of sensitized red cells, the tubes were incubated for 30 minutes at 37° C. and then examined. The Wassermann tests were carried out in an identical manner, the Wassermann antigen (prepared by Eagle's method) replacing the virus antigen in the mixture.

Sera.

Sera were inactivated at 56° C. for 30 minutes and then kept frozen at −23° C. Sera from Australian aborigines were obtained through the courtesy of Dr. J. A. R. Miles[1], Dr. R. J. Walsh[2], Dr. H. K. Ward[2], and Dr. W. R. Horsfall[3].

Complement-fixation tests with human sera.

Table 10 shows the results of complement-fixation tests with sera collected from Australian aborigines. The sera collected at Yuendumu and Katherine were used as pools, comprising respectively the sera collected from eighteen and ten natives. The Wassermann, HEV and normal suckling-mouse brain antigens were used. Non-specific reactions with the normal suckling mouse brain antigen were not observed.

TABLE 10.

Results of complement-fixation tests with aboriginal sera.

Sera from	No. giving reaction indicated				
	HEV− W.R.−	HEV strong+ W.R.+	HEV weak+ W.R.+	HEV− W.R.+	HEV weak+ W.R.−
Mona Mona	5	—	5	2	—
Yuendumu	5	—	1	2	—
Bungalon	4	5	5	—	2
Ooldea	7	—	2	—	—
Yuendumu pool	—	1	—	—	—
Katherine pool	—	1	—	—	—
Others	3	3	—	3	1
Total	24	10	13	7	3

HEV strong+=titre 1/40 or greater.
HEV weak+=titre 1/10 to 1/40.
HEV−=titre <1/10.
W.R.=Wassermann reaction.

It was formerly shown that HEV showed no reaction with sera collected from white patients with syphilis, infectious hepatitis or other diseases, and that complement-fixing antibodies could be detected in various hyperimmune-mouse sera to a titre of 1/160. The maximum titre obtained with the sera from aboriginal natives was 1/80, and a strong positive reaction was taken as 1/40 or greater. Although 45 p.c. of the aboriginal sera tested reacted with HEV, only 18 p.c. reacted strongly and all these also showed a positive Wassermann reaction; but not all sera showing a positive Wassermann reacted with HEV. The latter group comprised 12 p.c. of those tested.

Complement-fixation tests with sera from infected monkeys.

In order to maintain the H.HEV variant in its original form, preparations from infected suckling-mouse livers were given to monkeys by the oral route (see Section I). Sera were

[1]From the Institute of Medical and Veterinary Science, Frome Road, Adelaide, South Australia.

[2]From The Red Cross Blood Transfusion Service, York Street, Sydney, N.S.W.

[3]From the Department of Health, Commonwealth Health Laboratory, Cairns, Queensland.

collected at varying times after feeding the virus and tested for complement-fixing and neutralizing antibodies. The results of the complement-fixation and neutralization tests are given in Table 11, where it may be seen that a positive Wassermann reaction developed together with complement-fixing and neutralizing antibodies to both *N*.HEV and H.HEV. A prozone was observed in the Wassermann tests carried out with two of the high-titred monkey sera.

TABLE 11.

Summary of results obtained by feeding HEV to monkeys.

No.	Type	Inoculum	Bleedings[1] days after inoculation	Reciprocal of serum dilutions: Complement-fixing[2] antibodies HEV	Wa.[3]	Neutralizing antibodies	Virus demonstrated in faeces
62	*M. nemestrinus*	H.HEV	16	16	64	10	2 days at 10^{-3} dilution
		per os	27	64	128	15	3-6 days—trace
			34	8	128	>30	8-9 days—nil
63	*M. mulatta*	*N*.HEV	4	0	0	<10	
		per os	11	16	0	5	
			18	128	512	25	7 days—nil
			25	32	128	75	
			32	4	128		
			34	<4	64		
65	*M. irus*	H.HEV	0	0	0		
		per os	7	0	64		2-3 days at 10^{-1} dilution
			9	4	128		6-8 days—nil
			14	32	128		

[1]Blood collected by heart puncture.

[2]Complement-fixing antibodies to normal suckling-mouse brain antigen could not be detected at dilutions >1/10.

[3]Wa.=Wassermann antigen.

SUMMARY.

Complement-fixing antibodies to the hepato-encephalomyelitis virus are present in the sera of Australian aborigines. These sera also give positive Wassermann reactions, but do not react with normal suckling-mouse brain antigen. On the other hand sera giving a positive Wassermann reaction from white adults do not contain complement-fixing antibodies to HEV. Infection of monkeys by the oral route gives rise to the production of specific complement-fixing and neutralizing antibodies to HEV as well as to the production of a substance reacting with the Wassermann antigen to high titre. These findings are discussed in the following section.

V. DISCUSSION ON THE NATURE OF THE HEV.

It is the purpose of this section to discuss and summarize the experimental data presented in the four preceding sections and to compare the properties of the hepato-encephalomyelitis virus (HEV) with those of other infective agents, showing on preliminary examination some slight similarity to it.

The homogeneity of H.HEV and N.HEV.

When it was first shown that successive intraperitoneal passages of H.HEV-infected mouse brain resulted in a gradually increasing difficulty to demonstrate the OHE and jaundice and that, with the loss of these two properties, there was a much shorter incubation period, the possibility of there being present a mixture of two agents was immediately realized. During a period of eighteen months experiments were planned and carried out with this idea of demonstrating the presence of two infective agents. This was not accomplished, but the results provided further evidence that the variety of symptoms observed in infected mice was caused by variations of one agent. In view of the recent report by Niven *et al.* (1952), that the MHV, described by Gledhill *et al.* (1951), consists of a mixture of *Eperythrozoon coccoides* (the L component) and a virus (the S component), an attempt was made to separate HEV into two such components. This was not achieved and the presence of *E. coccoides* could not be demonstrated.

There are many properties which indicate the homogeneous nature of the H.HEV and *N*.HEV, viz.:

(a) The similarity of antigenic structure is clearly demonstrated by both complement-fixation and neutralization tests. Hyperimmune-mouse serum was prepared against *N*.HEV after ten successive passages on the chorio-allantois of developing chick embryos. It was shown that complement-fixing (see Stanley *et. al.*, 1953a) and neutralizing antibodies (Table 8) to both *N*.HEV and H.HEV were present in this serum to approximately the same titres.

(b) Neutralizing and complement-fixing antibodies to both *N*.HEV and H.HEV have been detected in the serum of the patient from whom the virus was isolated.

(c) Both *N*.HEV and H.HEV cause symptomless infections in monkeys (virus given orally) which result in the production of a substance reacting with the Wassermann antigen.

(d) HEV is primarily a "suckling-mouse virus," and both *N*.HEV and H.HEV cause fatal infections in infant mice, when given by the intradermal, intraperitoneal, intracerebral or oral routes. The apparent discrepancy in age susceptibility (see Table 4, Section 1) is less important when allowance is made for the difference in incubation periods of the variants (five days for *N*.HEV and twelve days for H.HEV).

(e) The pathological changes in brain and cord are identical with both variants.

(f) Both H.HEV and *N*.HEV behave similarly when studied by ultra-centrifugation and ultrafiltration (size approximately 100-150 mμ).

(g) Both agents produce pocks on the chorio-allantois following primary inoculation of 11-day old chick embryos.

(h) *N*.HEV arises as a result of laboratory manipulation of H.HEV.

The differences between *N*.HEV and H.HEV are:—

(a) The appearance of infected suckling mice.

(b) The apparent affinity of *N*.HEV for central nervous system tissue. This is illustrated by the ability of *N*.HEV preparations to infect adult mice by the intracerebral route following primary inoculation, while H.HEV cannot, as well as the marked clinical evidence of C.N.S. damage in suckling mice infected with *N*.HEV.

(c) Areas of liver necrosis are difficult to demonstrate with *N*.HEV, probably because of the early death following infection of the C.N.S. before lesions have fully developed.

When all these factors are taken into consideration, the most likely explanation of the findings is that *N*.HEV is a neurotropic variant of H.HEV which, through laboratory manipulation, has lost the ability to produce jaundice and OHE in suckling mice.

The association (if any) of HEV with aboriginal natives.

The finding that complement-fixing antibodies were present only in the sera of natives showing a positive Wassermann reaction indicated the likelihood of a non-specific reaction with the HEV complement-fixing antigen. This assumption was soon shown to be erroneous, but as an extra precaution the antigens were extracted with organic solvents in the approved fashion (de Boer and Cox, 1947). The fact that oral infection of monkeys results in the production of a substance reacting to high titre with the Wassermann antigen indicates that this reaction in aborigines requires further investigation. (At the moment, the view held is that such a reaction is usually the result of yaws or syphilis.)

The facts then may be summarized as follows:

(a) Specific complement-fixing and neutralizing antibodies are present in the sera of some aboriginal natives but not in any serum from the white population yet tested.

(b) Specific complement-fixing antibodies in aboriginal sera are associated with a positive Wassermann reaction. No complement-fixing antibody could be detected in the sera of syphilitic white people.

(c) No neutralizing or complement-fixing antibodies could be demonstrated in the sera of white patients with infective hepatitis, infectious mononucleosis, Weil's disease or homologous serum jaundice.

(d) Both neutralizing and complement-fixing antibodies were present in the serum from the aboriginal native from whom the virus was isolated.

These observations, together with the fact that the agent was excreted in the faeces of the aboriginal child, support the view that HEV infections may occur in Australian aborigines. If such a view were acceptable, the nature of

this infection is not yet known. Further evidence is needed to clarify the position, and at the moment attempts are being made to isolate further strains of HEV from the faeces of aboriginal natives in different parts of Australia.

Relationship of HEV to other infective agents.

The agents that will be considered are:

(a) the virus of infective hepatitis,
(b) a mouse hepatitis virus (MHV), recently described in a series of papers from the National Institute for Medical Research, London (Gledhill and Andrews, 1951; Gledhill *et al.*, 1952; Niven *et al.*, 1952),
(c) *Herpes simplex,*
(d) Theiler's group of viruses causing spontaneous mouse encephalomyelitis (Theiler, 1941),
(e) a murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin (Cheever *et al.*, 1949; Bailey, *et al.*, 1949),
(f) the Coxsackie group of viruses, and
(g) a mouse encephalomyocarditis virus (MEM).

(a) *The virus of infective hepatitis.* The sera of white patients with infective hepatitis were shown to contain no complement-fixing or neutralizing antibody to HEV. The agents behaved somewhat similarly in their resistance to germicides, but insufficient data were available on infective hepatitis to make an adequate comparison.

(b) *The mouse hepatitis virus (MHV).* In a previous communication (Stanley *et al.*, 1953a; see Table 4) the dissimilarities of HEV and MHV were pointed out. The outstanding differences were the inactivation of MHV by heat at 55° C., its inability to produce encephalitis and its ability to infect mice 21 days old. Furthermore, repeated attempts have resulted in failure to demonstrate *Eperythrozoon coccoides* either in HEV-infected mice or uninoculated mice.

(c) *Herpes simplex.* The virus of *Herpes simplex* was included for comparison because it readily caused a fatal infection of suckling mice when given by the intraperitoneal route. All strains of herpes isolated in this laboratory by suckling mouse inoculation gave rise to a blood-stained peritoneal exudate, and at post-mortem examinations lesions were found in the C.N.S. (These two properties are also shared by HEV when it is undergoing the H→*N* variation.) Both agents are approximately the same size. The viruses, however, differ in their susceptibility to heat and germicides, their ability to infect adult mice, and in the nature of the pocks produced on the chorio-allantois of the developing chick embryo.

(d) *Theiler's mouse encephalomyelitis viruses (TO, FA, GD-VII).* The possibility that *N.*HEV may be an encephalomyelitis virus latent in the mouse stock must of course be considered. HEV bears no antigenic relationship to this group of viruses and differs in size and ability to infect adult mice.

(e) *The murine disseminated encephalomyelitis virus (JHM).* This agent possesses physical properties quite distinct from HEV. It is susceptible to ether, heat at 50° C. for ten minutes or 0·25 p.c. formalin. It produces a different clinical picture from that of HEV infection and infects adult mice by different routes. It could not be isolated from blood or intestinal walls or contents.

(f) *The Coxsackie group of viruses.* The HEV is primarily a suckling-mouse virus and in this respect it is similar to the Coxsackie viruses. The Coxsackie viruses, although resistant to germicides (Stanley *et al.*, 1951), are not as markedly resistant as HEV. In almost all other respects HEV and the Coxsackie viruses differ, i.e., in histopathology, clinical picture and antigen structure.

(g) *A mouse encephalomyocarditis virus (MEM).* Personal communications from E. L. French indicated that a virus, similar in many respects to HEV, was present in the mouse stock of the Walter and Eliza Hall Institute of Medical Research, Melbourne. Hyperimmune sera prepared against the MEM strain contained specific complement-fixing antibodies to *N.*HEV. It has been found that myocardial lesions are not common with HEV, but have been noticed in a few of our sections. As yet, insufficient data are available on MEM to indicate whether it is identical with, or in what way it is related to, HEV. It does raise again the problem referred to in our first paper on HEV—that HEV is a latent virus present in our mouse stock. It is possible that some material present in the faeces of the aboriginal child stimulated the virus following intraperitoneal inoculation of suckling mice. There is a certain amount of evidence against this, viz.:

(i) The failure to demonstrate HEV following inoculation of other faecal suspensions in many thousands of mice.
(ii) The demonstration of antibodies in the serum of the patient from whom the virus was isolated.
(iii) The demonstration of complement-fixing antibodies in the sera of Australian aborigines with a positive Wassermann reaction.
(iv) The demonstration that monkeys infected with either variant developed a positive Wassermann reaction, and
(v) the demonstration that pooled serum from normal stock mice contained no detectable antibody to HEV.

The H→N variation.

The properties of the HEV are interesting from many points of view and some of our observations have been summarized in table form (see Table 12).

TABLE 12.

Some of the properties of the antigenically homogeneous variants of HEV.

Properties	*N*.HEV	H.HEV
Size	100-150 mμ	100-150 mμ
Survives heat at 60° C. for 30 minutes	+	+
Inactivated by 70 p.c. EtOH	+	+
Incubation period in suckling mice inoculated by I.P. route	5 days	10 days
Per os	5 days	10 days
Symptoms of infections in suckling mice	paralysis, tremors, inco-ordination, death	jaundice, OHE, inco-ordination, conjunctivitis, alopecia, death
Neutralized by *N*.HEV serum	+	+
Neutralized by H.HEV serum	+	+
Pathogenic for adult mice by I.P. route	−	−
Pathogenic for adult mice by I.C. route (on primary inoculation only)	+	−
Stimulates production of +W.R. in monkeys	+	+
Age susceptibility of mice by I.P. route	1-10 days	1-3 days
Pocks on the chorio-allantois	+	+
Predominant histopathological findings in suckling mice	encephalomyelitis	hepatitis, encephalomyelitis

I.P.=intraperitoneal. I.C.=intracerebral. +W.R.=positive Wassermann reaction.

More important from the laboratory point of view is the H→*N* variation. A study of this variation led to two important observations. One was the infection of suckling mice by the natural route employing the "contact" technique. The HEV "contact" mice are presumably infected *per os* from the faecal material or contaminated skin of the infected mouse placed in the litter. This technique may have some use in laboratory epidemiological studies of virus diseases. The other observation of interest was that a gastro-intestinal parasite could be converted into a neurotropic variant either quickly or slowly, depending on the nature of the host and the method of inoculation. Such a change, once complete, was shown to be irreversible. It is interesting to consider a possible analogy with viruses known to infect the C.N.S. of man. Those of the poliomyelitis group, and possibly the Coxsackie group, behave similarly in that they can be recovered from the intestinal tract and cause symptoms indicative of C.N.S. involvement. The question arises as to whether viruses like poliomyelitis are undergoing variations similar to those demonstrated for HEV.

SUMMARY.

Further properties of the hepato-encephalomyelitis virus are described. A description of the neurotropic variant (*N*.HEV) is given and the manner in which the change from a gastro-intestinal parasite (H.HEV) to a neurotropic one may be controlled and brought about by laboratory manipulation is also described. A study of the antibody content of aboriginal native sera revealed that complement-fixing antibodies were present in 18 p.c. of the sera tested. All sera reacting this way also reacted strongly with the Wassermann antigen. Monkeys infected with either variant developed a positive Wassermann reaction. HEV was shown to be highly resistant to heat and germicides. Filtration through gradocol membranes revealed a size of approximately 100-150 mμ. Mice infected with H.HEV were characterized by the production of copious faecal material of high fat content as well as by jaundice, oily hair effect, alopecia and incoordination. Both *N*.HEV and H.HEV produced pocks following primary inoculation of the chorio-allantois from infected mouse tissue. The pocks could not be demonstrated after four successive passages on the chorio-allantois. The properties of HEV are compared with those of other viruses, and it is thought to be distinct from any virus yet described. The possibilities of HEV being associated with infection in Australian aborigines are discussed.

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THE EXPERIMENTAL PRODUCTION OF PULMONARY OEDEMA IN UNANAESTHETIZED RATS BY MEANS OF INTRAVENOUS INFUSIONS OF RINGER-LOCKE SOLUTION, PLASMA AND BLOOD

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In previous work from this laboratory pulmonary oedema has been produced in rabbits by means of massive infusions of Ringer-Locke solution. The ease of inducing pulmonary oedema by this method has been investigated in normal rabbits, in rabbits subjected to anoxia and in rabbits given noradrenaline (Courtice and Korner, 1952; Korner, 1953). In these experiments the Ringer-Locke infusions produced about the same degree of blood volume expansion. Pulmonary oedema was more readily induced (1) with anoxia owing to cardiac failure, and (2) with noradrenaline owing to redistribution of blood secondary to systemic vasoconstriction. In the present experiments the effects of Ringer-Locke infusions on pulmonary oedema production have been compared with those of the more efficient blood volume expanders, blood and plasma.

METHODS.

Wistar strain rats were used in all experiments. Cannulation of one of their external jugular veins with polythene or transflex tubing was carried out under ether anaesthesia. The rats were allowed to recover from the anaesthetic and placed in special restriction cages (Bollman, 1948). Two hours after the operation, when the rat had completely recovered, transfusion was commenced. All fluids were administered through a small burette with a fine drip arrangement. The rate of administration of fluids varied from 1·5 ml./hr. to 12 ml./hr. The solutions used were sterile Ringer-Locke solution, and freshly collected homologous plasma and blood. In some experiments whole blood was used. In others, to prevent excessive haemoconcentration, the blood was diluted with fresh plasma to give a haemoglobin concentration of 50-60 p.c. All blood and plasma infused was cross-matched with the blood of the recipient. All experiments were of 5 hours duration.

Change in blood volume. In the case of Ringer-Locke and plasma, this was calculated from the change in the initial and final haemoglobin concentrations. In the case of the blood transfusions, the blood volume of the rats was calculated from the results of Montgomery (1951) and assumed to be 63 ml./kg. with a haematocrit of 42 p.c. From a knowledge of the haemoglobin concentration at the start and end of the experiment, and the haemoglobin concentration of the blood given, the changes in blood and plasma volume were calculated. All haemoglobin estimations were done in duplicate on a M.R.C. grey wedge photometer.

[1]This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

Method of killing rats, etc. At the end of the experiment the rats were killed by injecting 0·4 ml. of veterinary nembutal (60 mg./ml.) in 30 seconds. The animal was removed from its cage, the trachea tied and the amount of peritoneal and pleural fluid was measured. The lung and heart were dissected *en bloc* and the pulmonary vessels ligated. The lungs and a small portion of the trachea were placed on a watch glass and weighed on a laboratory balance to 0·05 gm. From this the lung/body weight ratio (weight of lung in gm./body weight in kg.) was calculated. Most lungs were immediately fixed in corrosive formalin and examined histologically.

RESULTS.

Normal lung/body weight ratio in rats.

In a series of 17 normal rats the mean lung/body weight ratio (L/B ratio) was 4·9 with a standard deviation of 1·5 and a standard error of the mean of 0·34. There was a statistically significant relationship between the lung weight and the body weight over the body weight range examined (180-340 gm.).

Lung/body weight ratio as an index of pulmonary oedema in rats.

In agreement with previous work in rabbits, the L/B ratio afforded a good index of the degree of pulmonary oedema present in the lungs. The normal L/B ratio varied rather more than in the rabbits (range 4·6). With L/B ratios from 6-7 there were distinct patches of pulmonary oedema, which was often interstitial, throughout the lungs. At L/B ratios from 7-8 the pulmonary oedema was more generalized, and above 8 there was usually a considerable amount of tracheal froth. The examination of a large series of histological sections showed that the microscopic appearance of oedema fluid in the lungs confirmed the macroscopic findings. With blood transfusions the lungs were always extremely congested and it was difficult to judge macroscopically whether pulmonary oedema was in fact present. However, there was good correlation between the degree of pulmonary oedema observed microscopically and the L/B ratios.

The distribution of infused fluid during transfusion with Ringer-Locke, plasma and blood.

The average values and standard error of the mean results are summarized in Table 1. With Ringer-Locke an average of 137 ml./kg. was infused over 5 hours. This resulted in a moderate degree of pulmonary oedema with a mean L/B ratio of 6·3. About half the fluid given was excreted as urine, and there was an average expansion of the blood volume of 14 p.c. With the plasma infusions, administration of half the volume of plasma compared to that given in the Ringer-Locke series, produced quite severe pulmonary oedema (mean L/B ratio 8·8). About 70 p.c. of the fluid infused was excreted in the urine, and there was a significantly greater increase in blood volume than in the Ringer-Locke series. With blood a similar picture was obtained as with plasma and a considerable amount of pulmonary oedema was produced.

TABLE 1.

Distribution of infused fluid following Ringer-Locke plasma and blood transfusions.

Experiment	No. of rats	Total fluid infused ml./kg.	Total fluid infused apart from red cells ml./kg.	Urine ml./kg.	Increase in blood volume p.c.	Total excess tissue fluid ml./kg.	Lung/ body weight ratio gm./kg.	Pleural fluid ml./kg.	Peritoneal fluid ml./kg.
Ringer-Locke	22	137±17	137±17	64±11	14±0·9	67±14	6·3±0·3	5·21±1·7	negligible
Plasma	15	70±7	70±7	51±7	45±8	1±8	8·9±0·95	4·0±1·3	0·4—0·2
Blood	13	73±4·6	53±3	53±5	49±3	−11±4	9·4±0·84	4·9±1·5	negligible

All figures quoted are the mean of the results and the standard error of the mean.

Analysis of factors affecting the formation of pulmonary oedema with transfusions.

In Fig. 1A, the L/B ratio has been plotted against the total excess tissue fluid plus 50 ml./kg./5 hr. (The total excess tissue fluid is the total fluid given minus urine produced minus increase in plasma volume.) The reason for adding 50 to the value for total excess tissue fluid was purely for convenience in the calculation of results, as this value was often negative with the plasma and blood transfusions. The L/B ratio rose fairly gradually with the Ringer-Locke infusions, rising on the average by 2·3 for an increment in the total

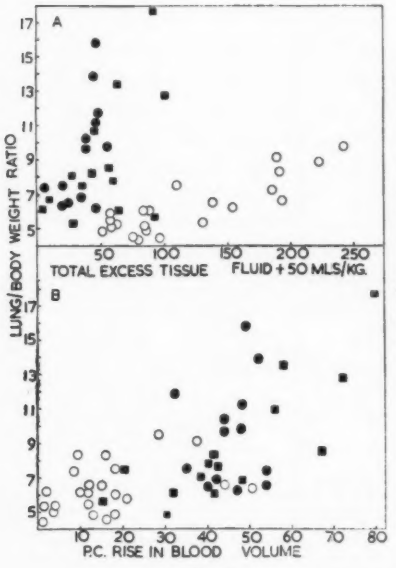

Fig. 1.

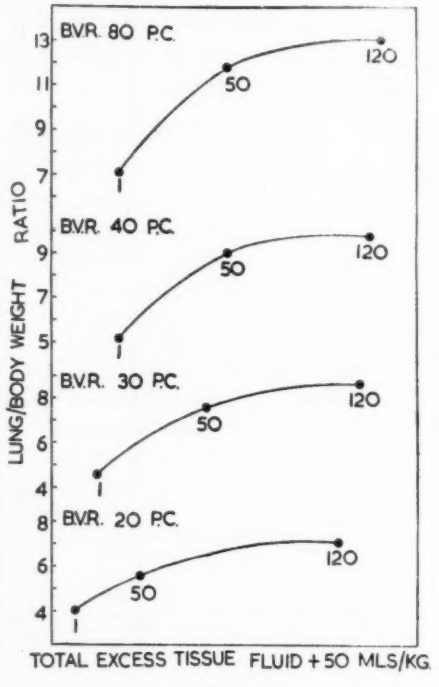

Fig. 2.

Fig. 1. A/Relation between L/B ratio and total excess tissue fluid plus 50 ml./kg. B/Relation between L/B ratio and rise in blood volume (p.c.).

O—Ringer-Locke infusions;
■—Plasma infusions;
●—Blood infusions.

Fig. 2. Effect of increasing total excess tissue fluid plus 50 ml./kg. on the L/B ratio at different levels of blood volume expansion. The points on each line represent different amounts of excess tissue fluid plus 50 ml./kg. BVR=Blood volume rise (p.c.). For further explanation see text.

excess tissue fluid of 100 ml. The relationship was quite different with the blood and plasma transfusions, the L/B ratio rising much more abruptly. In Fig. 1B the L/B ratio has been plotted against the rise in blood volume produced by all the infusions. With an increase in blood volume above about 40 p.c. there was a rapid increase in the degree of pulmonary oedema produced. It is evident from Fig. 1 that the degree of pulmonary oedema was related to both the excess tissue fluid and the rise in blood volume. In order to appreciate the relative importance of these two factors during Ringer-Locke infusion on the one hand, and administration of blood and plasma on the other, two multiple regression equations were derived from the data.

With Ringer-Locke infusions, the L/B ratio was significantly related to the total excess tissue fluid, but there was no significant effect owing to the expansion of the blood volume. This is summarized in equation (1):

$$\text{Log LB} = 0{\cdot}784 + 0{\cdot}385\ (\log \text{TETF}_{50} - 2{\cdot}026) + 0{\cdot}048\ (\log \text{BVR} - 1{\cdot}142 \quad \ldots\ldots \quad (1)$$

where LB = Lung/body weight ratio (gm./kg.)

TETF_{50} = Total excess tissue fluid plus 50 ml./kg.

BVR = Rise in blood volume p.c.

The first regression coefficient is statistically significant ($p = 0{\cdot}001$); the second one is not ($p = 0{\cdot}15$).

The lack of significance of the blood volume factor in this series is probably only due to the relatively small numbers of experiments used. The probability of 0·15 for this factor may be regarded as suggestive of its possible significance if the experiment were enlarged.

In the case of the plasma and blood transfusions, it is obvious from Fig. 1 that the results with these two fluids are very similar. Accordingly the results have been pooled in deriving equation (2):

$$\text{Log LB} = 0{\cdot}936 + 0{\cdot}123\ (\log\ (\text{TETF}_{50} - 1{\cdot}556) + 0{\cdot}440\ (\log \text{BVR} - 1{\cdot}647) + 0{\cdot}130\ (\log \text{HbF} - 1{\cdot}963) \quad \ldots\ldots \quad (2)$$

where LB = Lung/body weight ratio (gm./kg.)

TETF_{50} = Total excess tissue fluid plus 50 ml./kg.

BVR = Rise in blood volume, p.c.

HbF = Final haemoglobin concentration, p.c.

The first regression coefficient is statistically significant ($p = 0{\cdot}05$)

The second regression coefficient is statistically significant ($p < 0{\cdot}01$)

The third regression coefficient is not statistically significant ($p = 0{\cdot}5$).

The above equation indicates that the L/B ratio rises with an increase in total excess tissue fluid and also with rise in blood volume. The last term of the equation, the final haemoglobin concentration, was introduced to allow for any possible effect due to viscosity in the experiments with blood compared with

those with plasma. In this series of experiments, the effect of change in viscosity, though numerically in the right direction, was not statistically significant. The results of equation (2) are shown graphically in Fig. 2. Each line shows the variation in L/B ratio for different amounts of total excess tissue fluid plus 50 ml./kg. ($TETF_{50}$) at a constant level of blood volume rise. The points on each line represent different amounts of $TETF_{50}$. It may be seen that with a blood volume rise of 20 and 30 p.c., the L/B ratio increases rather gradually over the whole range. With a blood volume rise of 40 to 80 p.c., small increments in $TETF_{50}$ produce a much steeper rise in the L/B ratio than with smaller blood volume increases[2].

Serious effusions.

Transfusion with the various fluids resulted in the accumulation of small amounts of pleural fluid as was seen in Table 1. There was almost no free fluid in the peritoneal cavity. In relation to the origin of the pleural fluid in cases with pulmonary oedema, it has been suggested that the pleural fluid arises as a transudate through the thin pleura from the lung engorged with oedema fluid (c.f. Richter, 1952). In order to throw some further light on this question, the following regression equation was derived from the results of the Ringer-Locke infusions, relating pleural fluid to the L/B ratio and to the total excess tissue fluid:

$$PF=5\cdot2+1\cdot22\ (LB—6\cdot2)+0\cdot093\ (TETF—67) \quad \ldots\ldots \quad \ldots\ldots \quad (3)$$

where PF is pleural fluid (ml./kg.)

LB=Lung/body weight ratio (gm./kg.)

TETF=Total excess tissue fluid (ml./kg.)

The first regression coefficient is not significant ($p=0\cdot6$)

The second regression coefficient is significant ($p=0\cdot01$).

The results of the equation indicate a better degree of correlation between the amount of pleural fluid and the total excess tissue fluid than between the former and the L/B ratio. The significance of this will be considered below.

DISCUSSION.

Production of pulmonary oedema by Ringer-Locke, plasma and blood.

Striking differences were observed in the distribution of fluid with the three agents used in the present experiments. There was a much greater blood volume expansion with blood and plasma than with Ringer-Locke, probably because of the greater resistance offered by the capillary membrane to the passage of the protein-rich fluids. A much greater proportion of fluid was excreted as urine with blood and plasma, and in fact very little oedema fluid accumulated in any

[2]It is clear that even with no excess tissue fluid retained (i.e., $TETF_{50}=50$ ml./kg.), a blood volume expansion above 40 p.c. results in a significant production of pulmonary oedema.

of the tissues except the lungs and pleural cavities. The average amount of fluid in the lungs was nearly double that accumulating with Ringer-Locke. From the results of equation (2) and Fig. 1B it would seem that the greater ease of inducing pulmonary oedema with plasma and blood was almost entirely due to their efficiency as blood volume expanders. By substituting into equation (2), based on the results with blood and plasma, the average values for total excess tissue fluid and blood volume expansion obtained with Ringer-Locke infusions, the L/B ratio calculated is 6·02, in good agreement with the actual mean of 6·3 obtained with the Ringer-Locke infusions. The general hypervolaemia thus apparently leads to an increase in pulmonary blood volume and pulmonary vascular bed. If the blood volume is raised above 40-50 p.c. there is probably a rapid increase in the pulmonary capillary pressure as judged by the rapid formation of pulmonary oedema. The reasons for the greater effectiveness of blood and plasma transfusions in producing pulmonary oedema would thus appear to be purely haemodynamic.

Species differences in fluid distribution following Ringer-Locke infusions.

Fig. 3 shows the mean effects obtained with the Ringer-Locke infusions in rats and the results of similar experiments in rabbits (Courtice and Korner, 1952; Korner, 1953). It is evident that pulmonary oedema is produced more readily in rats than in rabbits. The rate of accumulation of pulmonary oedema (i.e., the slopes of the regression lines) once pulmonary oedema begins to form are similar in both groups. Courtice and Korner (1952) suggested that massive intravenous infusions in rabbits led to an increase in pulmonary blood volume and expansion of the pulmonary vascular bed. Because of the expansibility of the pulmonary vascular bed and the high osmotic pressure reserve of the plasma, very large net infusions were required to raise the pulmonary capillary pressure sufficiently to produce pulmonary oedema in rabbits. From our present results it would seem that the degree of expansibility of the pulmonary vascular bed is more limited in the rat than in the rabbit, and that a rise in pulmonary capillary pressure is obtained more readily. This fact may account for the greater ease of producing pulmonary oedema in rats than in rabbits by other methods (viz., NH_4^+ pulmonary oedema—Koenig and Koenig, 1949; Cameron and Sheikh, 1951). The distribution of fluid in the serous cavities was also different in rats

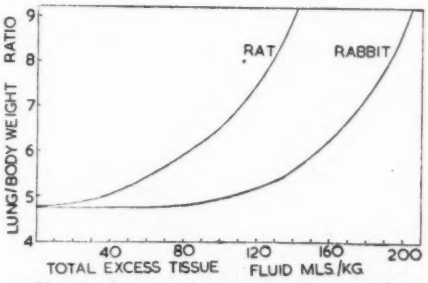

Fig. 3. Relation between total excess tissue fluid and L/B ratio following 5 hours infusion of Ringer-Locke in rats and rabbits.

and rabbits. Almost all the fluid in the serous cavities was present in the pleural cavity in rats, whilst in the great majority of rabbits it was in the peritoneal cavity.

Source of pleural fluid.

Richter (1952) has suggested from his results with α-naphthyl thiourea in rats, that the pleural fluid under these conditions overflows, as it were, through the thin pleura from the lungs. In the present experiments the evidence on the whole suggests that during Ringer-Locke transfusion this is not the case. The results of equation (3) showed a much better degree of correlation between the amount of pleural fluid and the total excess tissue fluid than between the amount of pleural fluid and the L/B ratio. This argument is, of course, not conclusive evidence that the pleural fluid does not arise in the lungs. However, there was no significant difference in the amount of pleural fluid resulting from the blood and plasma transfusions compared with the amount produced by the Ringer-Locke infusions, although there was much greater accumulation of pulmonary oedema with the former group. Assuming the rate of lymphatic removal to be fairly similar in all three types of transfusion, it would appear that the pleural fluid arises from extrapulmonary sources.

SUMMARY.

Pulmonary oedema was produced much more easily by means of blood and plasma transfusions than by Ringer-Locke infusions. The reason for this depends on the much greater efficiency of blood and plasma as blood volume expanders. Above a rise in blood volume of 40-50 p.c., considerable amounts of pulmonary oedema were produced.

Pulmonary oedema was induced much more readily in rats, by means of prolonged infusions of Ringer-Locke solution, than in rabbits. The expansibility of the pulmonary vascular bed was thought to be more limited in the rat than in the rabbit.

The possible source of origin of the pleural fluid resulting from the transfusions has been discussed.

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THE SEPARATION OF A CYTOCHROME-CONTAINING FRACTION FROM *AEROBACTER AEROGENES*[1]

by F. MOSS[2]

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(Accepted for publication 21st December, 1953.)

The experiment described in this paper arose from an attempt to separate the cytochromes of *Aerobacter aerogenes.*

It was reported by Tissières (1952) that cytochrome a_2 could not be demonstrated in a soluble preparation prepared by centrifugation of glass-ground *A. aerogenes,* although cytochromes a_1 and b_1 were present. An ammonium sulphate precipitate prepared from this soluble extract showed strong bands of a_1 and b_1, but a_2 could not be demonstrated even at the temperature of liquid air.

The a_2-free suspensions and the resuspended precipitate showed respiratory activity. It was suggested that either a_1 and b_1 were able to function without the oxidase a_2, or that the latter may be present in an amount too small to be demonstrated. The experiment here described supports the latter suggestion.

Schachman *et al.* (1952) found that extracts prepared from a number of bacterial species, including *Escherichia coli,* which were disintegrated by grinding, sonic disintegration, and phage lysis, exhibited similar ultracentrifugal patterns, from which it was concluded that these particles were not an artefact due to disintegration, but were present as such in whole cells. The fraction from *E. coli* analogous to the cytochrome-bearing fraction of *A. aerogenes,* described in the present paper, may have been discarded in the experiment of Schachman *et al.* in a fraction separated at 7,000 r.p.m. for 10 minutes.

Strittmater and Ball (1952) found that the microsomes of rat liver contained only cytochrome b_1, while cytochromes a, b and c were present in the mitochondria.

Separation of the cytochromes of *A. aerogenes* into different particulate fractions could not be achieved in the experiments described in the present paper. Cytochromes a_2, a_1 and b_1 have been found together in a particulate fraction of the cell.

[1]This work was carried out with the aid of a grant from the National Health and Medical Research Council of Australia.

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Materials and Methods.

The organism. The strain of *Aerobacter aerogenes* used in this experiment occurred as a contaminant in a culture inoculated with *Escherichia coli*. The contaminant rapidly outgrew the *E. coli*. It showed exceptionally strong absorption bands of cytochrome b_1, a_1 and a_2 at 555, 590 and 630 $m\mu$ respectively. The medium used throughout this experiment was a glucose ammonium-phosphate medium with 5 p.c. broth.

Experimental procedures.

The bacteria were grown with aeration for 17 hours.

Disintegration was produced by a Raytheon 9 K.C. magnetostriction oscillator in a nitrogen atmosphere at −2° C. In experiment 1, disintegration was carried out on 40 ml. amounts of cell suspension in water containing 0·1 gm. per ml. of bacteria (dry weight) for 20 minutes, and in experiment 2 on 20 ml. amounts.

Centrifugation was carried out under N_2 with the rotor initially at −5° C. in a Servall centrifuge. Fractions were removed as follows: D_1, after 1,000×*g*. for ½ hour; D_2, after 20,000×*g*. for 1 hour; D_3, after 50,000×*g*. for 1 hour; S_3 was the supernatant.

Extraction of the haemins was carried out as follows: Each fraction was extracted twice with 1 p.c. HCl in acetone. After each extraction the cell material was separated by centrifugation and finally dried at 105° C. and weighed. The extracted haems were then taken into ether by dilution with 1 p.c. aqueous HCl. The ether phase was washed with 1 p.c. HCl and the haemins extracted with M/20 NaOH. 20 p.c. pyridine was added. Estimations of the protohaemochrome were carried out spectrophotometrically at 555 $m\mu$ immediately after the addition of dithionite. ϵ mol. was taken at 31,000 and the molecular weight of protohaem as 619. These estimations were found to vary not more than 8 p.c. of the mean, using as little as 0·2 gm. dry weight of cells.

An attempt was made to compare the amount of b_1 and a_2 in the various fractions with that in the whole cells. Whole cell paste and disintegrated material were placed in glass wedges and the spectra observed simultaneously, using a microspectroscope. The light intensity was adjusted until background illumination was similar. The thickness of the wedges in the optical path was measured when absorption was equal, as in the wedge-trough method of Keilin and Hartree (1946).

The amount of material in each fraction was estimated also by the biuret method (Strickland, 1951), which was in agreement with the weights of the dried residue of the acetone-HCl extract.

RESULTS.

The D_1 fraction consisted of partly disintegrated or whole cells. D_2 consisted mainly of particles of from 0·03μ to 1·0μ (mode 0·06μ), together with a few unbroken cells. Both these fractions were buff coloured and opaque. The D_3 fraction was translucent and varied in colour from red to nearly black. The diameter of particles was from 0·02μ to 0·07μ (mode 0·04μ). S_3 was a yellow liquid which showed the Tyndall effect. This fraction contained particles <0·02μ—0·04μ (mode <0·02μ). Measurements of particles were made from electronmicrographs of formalin-fixed material, kindly prepared by Mr. V. Pickles, of the University of Technology, Sydney.

D_2, which had been washed and recentrifuged until free of D_3 material, again produced material similar to D_3 after a further period of disintegration.

The absorption bands of cytochromes b_1, a_1 and a_2, which were most intense in the D_2 fraction, were present also in the other fractions.

In the S_3 fraction the a_2 band was visible in a 10 cm. layer. Below this length the b_1 band only was visible. When the spectrum from 10 cm. of S_3 was compared with a layer of whole cell paste as described, the a_2 and b_1 bands showed the same relative intensity as in the other fractions. No cytochrome bands were seen after centrifugation at 50,000×*g.* for 4 hours in the S_3 fraction. The absorption bands of a_2 and b_1 in the D_2 and D_3 fractions also appeared to be of the same relative intensity in each fraction.

Desoxycholate, cetyl trimethyl ammonium bromide and "teepol"[3] brought about dispersion of most of the D_2 material.

The absorption spectrum of the pyridine haemochromes extracted from *A. aerogenes* showed maxima at 420, 524 and 555 mμ, which correspond to those of protohaemachrome, and at 613 mμ which corresponds to the haemochrome from cytochrome a_2 (Barrett and Lemberg, in the press). The ratios of the heights of the maxima 420 : 524 : 555 : 613 being 8·3, 1, 1·4, 0·3 respectively. The band at 613 mμ, however, was very variable.

TABLE 1.

Protohaem content of fractions of A. aerogenes.

Fraction	Protohaem (μg. per gm. dry weight), percentages of total protohaem in brackets		Dry weight (gm.) percentages in brackets	
Experiment	1	2	1	2
P. (whole cells)	0·38	0·23	1·21	0·96
D_1 (cell debris)	0·39 (42·5)	0·20 (4·9)	1·74 (38·5)	0·20 (6)
D_2 (20,000×*g.*)	0·66 (44·5)	0·47 (74)	1·06 (23·5)	1·38 (41)
D_3 (50,000×*g.*)	0·51 (6·4)	0·27 (15·1)	0·19 (4)	0·46 (13)
S_3 (soluble fraction)	0·065 (6·6)	0·04 (6·0)	1·55 (34)	1·34 (40)
Recovery relative to that of P fraction	100 p.c.	93 p.c.	94 p.c.	89 p.c.

As shown in the Table, the D_2 fraction comprised 23·5 p.c. and 41 p.c. of the total dried material in experiments 1 and 2 respectively, and the protohaem concentration was about twice that of the original whole cells.

In experiment 2, disintegration was more effective because smaller volumes were submitted for each sonic treatment.

The amount of protohaem present in whole cells varied from 0·1—0·4 μg. per gm. dry weight in the several experiments from which the results in Table 1 were selected.

[3] A sulphonated paraffin prepared by the Shell Co.

In the soluble S_3 fraction, 34 p.c. and 40 p.c. of the cell material were present in experiment 1 and 2 respectively. This fraction contained only about 6 p.c. of the total protohaem.

Further attempts to purify the fractions were not made because of the instability of the cytochromes. This experiment was performed several times in an air atmosphere instead of in nitrogen as described. It was found that the haem recovered from the disintegrated fractions in air was as low as 20 p.c. of that from whole cells in contrast to almost 100 p.c. in the experiments made nitrogen-free. This may be due to the oxidation of haems by hydrogen peroxide, which is known to be produced during sonic treatment.

DISCUSSION AND CONCLUSIONS.

Separation of cytochrome a_2 and b_1 into different particulate fractions has not occurred, as is shown by the constancy of the relative amounts of cytochrome a_2 to b_1 in each fraction prepared from the same original cells.

The D_3 fraction probably contains mostly the same material as D_2 at a stage of further disintegration, but the fact that its protohaem content is lower than in D_2 indicates an admixture of other material free from haem.

The variation in the amount of protohaem in different whole cell preparations may have been caused by variation in oxygen tension during preparation of the cultures. The dependence of cytochrome a_2 on oxygen tension has been described in a previous paper (Moss, 1952).

It is concluded that the cytochromes occur together in a cell fraction which, under the conditions of this experiment, is all deposited at $50{,}000\times g$. and which comprises more than half of the cell substance. If individual cytochromes are associated with separate particles, it is likely that they are less than $0{\cdot}02\mu$ in diameter.

The fatty nature of this insoluble fraction is demonstrated by its dispersion by detergents, and the observation (not here described) that Sudan black III was taken up by this fraction of the cell.

Whether any of the particulate fractions described in this experiment correspond to morphological units within the cell, or whether the particles are an artefact produced by fragmentation of a continuous matrix cannot be decided.

SUMMARY.

A. aerogenes disintegrated by sonic means was separated by centrifugation at 20,000×*g.* into particles of 0·03μ to 1·0μ diameter, at 50,000×*g.* into particles of 0·02μ to 0·07μ and a soluble fraction.

The particulate fractions comprised just over 50 p.c. of the disintegrated cell and contained almost 90 p.c. of the protohaem.

The soluble fraction contained about 40 p.c. of the cell material and contained 6 p.c. of the protohaem.

Separation of individual cytochromes has not been achieved.

Acknowledgments. I wish to thank Dr. R. Lemberg, F.R.S., for his continued advice and interest. I wish to thank also the Rockefeller Foundation for the gift of the Raytheon magnetostriction oscillator and the Servall centrifuge used in this experiment.

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OBSERVATIONS ON INFECTION OF MICE WITH *EPERYTHROZOON COCCOIDES* SCHILLING

by E. H. DERRICK, J. H. POPE, S. K. CHONG, J. G. CARLEY AND P. E. LEE

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This paper reports the presence of the parasite *Eperythrozoon coccoides* in the blood of laboratory mice in Brisbane and some observations that have been made on it.

In 1948 white mice were obtained from various sources in Brisbane for certain investigations then being made and it was noticed that a majority of the mice from two of the sources had enlarged spleens. In some cases the spleen measured more than 25×8 mm. and its weight exceeded 2 p.c. of the body weight.[1]

The splenomegaly in these colonies was traced to infection with *E. coccoides* when Giemsa-stained smears of blood were examined after splenectomy.

In 1949, a breeding stock of mice was established at this Institute from mice kindly supplied by Sir Macfarlane Burnet, Director of the Walter and Eliza Hall Institute, Melbourne, and after this stock multiplied, the mice used in our work were drawn from it. These mice are of many colours ranging from white to black. They are large—the usual weight at three weeks being 12 to 16 gm. Older mice often become obese and reach a weight of 60 to 100 gm. If not obese, the maximum weight is about 45 to 55 gm. Spontaneous splenomegaly has been seen in them only very occasionally.

A leading aim of our work has been the isolation of infective agents from human, animal and arthropod sources by means of mouse inoculation. A number of agents have been thus obtained and maintained in mouse passage until their identification. In August, 1951, the occurrence of splenomegaly in two series of mice, one inoculated from mites (*Laelaps* sp.) and one from ticks (*Ixodes tasmani* Neumann), aroused hopes that some agents pathogenic for mammals had been obtained from the arthropods. However, blood examinations after splenectomy showed the presence of *E. coccoides*.

The source of these two infections is obscure. It is possible that *E. coccoides* had remained in the experimental mouse room in some unsuspected way since our earlier work with it. Perhaps it came in from some extraneous source. It is very unlikely that the source was in our stock mice. Although the blood of only 19 has been studied after splenectomy, hundreds of mouse to mouse transfers have been made under conditions that would have disclosed a transmissible splenomegaly, and only on these two occasions to our knowledge has *E. coccoides* intruded.

[1]Normal linear measurements are discussed later. Our figures for the spleen/body weight of uninfected mice usually lie between 0·2 and 0·5 p.c.

There is a preliminary report in the literature (Eliot, 1936) (we have not been able to trace the detailed study) that attributes transmission of *E. coccoides* to the mouse louse *Polyplax serrata* (Burmeister). There were certainly numerous lice on the two colonies of mice which were noted in 1948 to be commonly infected with *E. coccoides*. We think that our own stock is free from lice. We have not seen any of them in recent years and the periodic dipping with gammexane suspension that is used in an attempt (only partially successful) to control acarine parasites—*Myobia muris-musculi* (Schrank), *Radfordia affinis* (Poppe) and *Myocoptes musculinus* (Koch)—would appear to be highly effective against lice. Dingle (1941), in his review, notes that attempts to induce infection by other vectors than *P. serrata*, by contact, by the oral route and by hereditary transmission have all been unsuccessful.

Splenomegaly as an index of infection.

In the routine examination of inoculated mice the spleen is studied carefully, for, in non-fatal and non-focal infections, splenomegaly may be the only macroscopic sign that some infection is present. The length and breadth of the spleen are measured in millimetres and their product used as an index of size. There is considerable variation in the sizes of both normal and abnormal spleens, and much overlap between them, but a product of over 100 may be regarded provisionally as abnormal and as an indication for further investigation. (In special cases the spleen was weighed, which gave a more accurate index of size.)

TABLE 1.

Spleen sizes of various series of mice.*

	A Uninoculated mice	B Mice examined 7-28 days after inoculation with presumably uninfected material	C Mice examined 7-28 days after inoculation with *E. coccoides*
Number of observations	100	100	100
Highest index	117	137	176
Lowest index	45	36	75
Mean index	76·9	79·8	129·35
Standard deviation of mean	12·46	18·99	22·68
Number of spleens with index over 100	3	13	93

* The index of size used in the Table is the product of length and breadth in millimetres.

Table 1 compares the sizes of spleens of (A) normal mice of our stock, (B) mice which had been inoculated intraperitoneally with presumably non-infective material 7–28 days before examination, and (C) mice inoculated intraperitoneally with *E. coccoides* 7-28 days before examination. The weights of these mice lay between 25 and 45 gm.; the mean spleen size is only slightly greater at 45 gm.

than at 25 gm. Each of the series in Table 1 consisted of equal numbers of male and virgin female mice; between the sexes the difference in spleen size was negligible.

Between Series A and B, the difference of means is not significant, but there is a significant excess of cases in B with a spleen index over 100. Possible explanations for this include a non-specific reaction to inoculated tissue or the presence of an unrecognized infective agent in some inocula. Most mice in B had been inoculated with liver-spleen taken from apparently healthy mice. Between Series A and C the difference of means is 20 times the standard error, and between B and C 17 times.

The spleen in Eperythrozoon infection.

The only macroscopic abnormality observed in infected mice was enlargement of the spleen. Although there was wide variation in individual cases, the splenomegaly was usually obvious by the seventh day after inoculation and reached its maximum in the third week (Fig. 1). Thereafter the size declined, at first rapidly and then gradually.

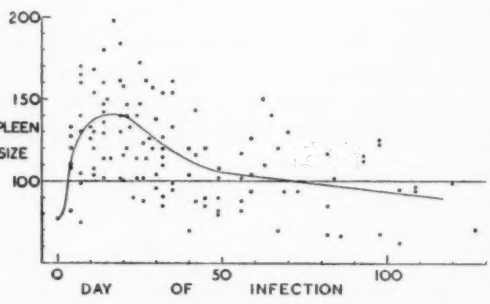

Fig. 1. Size of spleen (length×breadth in mm.) in cases of infection with *E. coccoides* examined at various intervals after inoculation.

The enlarged spleen was dark red and swollen, with rounded edges. On the cut surface, the Malpighian corpuscles stood out prominently against the dark red background.

Histologically, an intense congestion of the red pulp of the spleen was already present four days after inoculation; it persisted for three or four weeks and then became less evident. There was also a considerable increase in the amount of lymphatic tissue in the spleen. The Malpighian corpuscles enlarged and adjacent ones often coalesced. During the most active phase of the infection they contained large germinal centres with many cells in mitosis.

No histological changes referable to *E. coccoides* infection were observed in organs other than the spleen.

An instructive incident occurred when, in the performance of splenectomy on a male mouse weighing 30 gm., a thin slice of the spleen was accidentally left behind. The portion removed weighed 0·055 gm.; the piece left behind was estimated to weigh about 0·005 gm. No blood parasites appeared during the next fortnight. The mouse was then inoculated subcutaneously with mouse blood containing *E. coccoides*, and the parasite became very numerous in the blood six to eight days later. The amount of the spleen left behind, estimated at

1/12 had, therefore, not been sufficient to prevent gross multiplication of *E. coccoides*. When the mouse was sacrificed 14 days after infection, and 28 days after splenectomy, the spleen fragment had increased about 40-fold to 0·2 gm. and had become over three times as large as the original spleen.

Titration of tissues.

Three unsplenectomized mice were sacrificed 14 days after inoculation. Heart blood was taken from each mouse, received into 3·8 p.c. sodium citrate solution and pooled. Dilutions up to 10^{-8} were made in serum saline (10 p.c. horse serum in physiological saline) and three mice inoculated intraperitoneally with 0·5 ml. of each dilution. Infectivity of inocula was judged by splenomegaly in the mice when examined after 14 days.

All the dilutions tested were positive, which indicates that eperythrozoa may be quite numerous in the blood during the carrier stage, although they are rarely demonstrable then in blood films.

A second titration experiment included a range of tissues to seek evidence on the distribution of the organism. Three mice were used and the blood treated as before. Portions of other tissues were pooled, weighed, ground with pestle and mortar and alundum and diluted to 10^{-1} with serum saline. They were allowed to stand for an hour in the refrigerator, when the supernate was drawn off for the preparation of further dilutions. Because of the lability of eperythrozoa, all dilutions were kept in the refrigerator until inoculated and undue delay was avoided.

TABLE 2.

Titrations of organs for infectivity.

	First experiment	Second experiment	Third experiment
Blood	10^{-9}	10^{-8}	10^{-7}
Brain		10^{-5}	
Heart		10^{-9}	10^{-7}
Lung		10^{-6}	
Liver		10^{-5}	
Spleen		10^{-6}	10^{-7}
Kidney		10^{-6}	
Thigh muscle		10^{-5}	

The results (Table 2) show a fairly even distribution of infectivity in the different organs, probably dependent on the blood content. No attempt had been made to wash the tissue samples free from blood. A suggestion that there might be some concentration of organisms in the heart was not confirmed at a third titration.

Duration of infection with E. coccoides.

The duration of infection was studied in two ways. One series of mice was splenectomized at intervals and the blood examined for eperythrozoa. (It was

noted that, in mice examined late in the course of their infection, the eperythrozoa did not show the predilection for polychromatic cells that is a feature in the early stage.) Another series was examined by subinoculation of blood or tissues into further mice, the size and appearance of their spleens after 14 to 21 days being used as an indication of infection. This method is not specific, but under the conditions of the experiment usually gave definite results, especially when several mouse to mouse transfers were carried out. Occasionally, as at Day 196, the result was equivocal.

TABLE 3.

Duration of infection with E. coccoides.

Interval between inoculation and examination (days)	Demonstration of eperythrozoa after splenectomy	Splenomegaly in subinoculated mice
56		+, +
63	+, +	+
65		−
66	+, +	
70		+
84		+
86	−, −	
98		+, +
109	+, −	
117, 120, 120		−, −, −
127		+
143		−
146		+, −
148	−, −	
168, 170, 170		−, −, −
196		?
265, 273, 273		−, −, −
288	−, −, −, −	−, −, −, −
312		−
428		−, −

The two methods gave similar results (Table 3). The infection was still present in all mice but one examined up to twelve weeks after inoculation, and in some of those examined during the next nine weeks. The longest proved duration in our series was 146 days.

The duration of infection in splenectomized mice was not investigated. Dinger (1929) records that in them eperythrozoa may be seen in the blood in small but variable numbers for up to six months.

An attempt was made to reinfect four mice. When they were investigated at 288 days after their first inoculation, no evidence of persisting infection was found by either method. Splenectomy was performed and blood smears examined on five days a week for three weeks without disclosing eperythrozoa. Each excised spleen was inoculated into mice, but no splenic enlargement was found in them or in mice of two further passages.

Twenty-one days after splenectomy (309 days after the original inoculation) the four mice were reinoculated with *E. coccoides*. Blood smears were examined for three weeks as before, but no eperythrozoa were observed. Thirty-five days after re-inoculation the test mice were sacrificed and citrated heart blood from each inoculated into three mice, one of each group being splenectomized; no eperythrozoa were seen in the blood of the splenectomized mice, nor did splenomegaly develop in the others.

Thus no evidence was obtained that the four test mice were successfully reinfected.

Other observations.

Numerous unsuccessful attempts were made to cultivate *E. coccoides* in embryonated eggs. When it was inoculated into the yolk sac it survived for three to nine days as determined by reinoculation of mice. But attempts to carry it through a second passage failed. Similarly, it survived for six days after inoculation into the amniotic cavity, but further passage failed. Inoculation on to the chorio-allantoic membrane was also unsuccessful.

Two attempts to infect guinea-pigs failed.

SUMMARY.

Eperythrozoon coccoides has been found in laboratory mice in Brisbane. Its presence can be a source of confusion in attempts to isolate infective agents in mice.

Observations were made on the course of the experimental infection in unsplenectomized mice.

The infection is characterized by splenomegaly, which is well marked from 7 to 28 days after inoculation. Histologically, the spleen shows hyperplasia of the lymphatic tissue and congestion of the red pulp.

At 14 days after inoculation, a high titre (10^{-6} to 10^{-8}) of infectivity was found in the blood, although eperythrozoa are rarely seen in blood smears of infected unsplenectomized mice. No concentration of infectivity was found in other organs or tissues.

The infection appears to be self-limited and to terminate naturally after about 12 to 21 weeks.

An attempt failed to reinfect four mice ten months after their original inoculation.

Acknowledgments. We desire to thank Misses V. M. Pask and M. McCaffrey for valued assistance with the egg inoculations, and Mr. R. Domrow in the identification of mice ectoparasites.

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THE METABOLISM OF OYSTER SPERMATOZOA

2. THE EFFECT OF MALONIC ACID AND ESTERS OF SUBSTRATES[1]

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(Accepted for publication 21st December, 1953.)

It has previously been shown (Humphrey, 1950) that metabolically active preparations of oyster sperm can be obtained by making sea-water extracts of mature gonad tissue, and that such suspensions show increased O_2 consumption in the presence of α-ketoglutaric, oxalacetic, pyruvic and aminoacetic acids, but not lactic, succinic, fumaric, malic and glutamic acids, glycogen, glucose, fructose or lecithin.

Beevers *et al.* (1952) have shown that, in plant cells, many acids do not enter unless they are added as esters. In this present study, the effect of such esters on the O_2 uptake of oyster sperm has been investigated.

Further, Barron and Goldinger (1941) showed that malonic acid *increases* the O_2 uptake of sea-urchin sperm. This effect has been confirmed with oyster sperm, and the action of malonic acid in the presence of various substrates has also been investigated.

METHODS.

In general, the methods previously given (Humphrey, 1950) were used. In addition, *iso*-citric acid was prepared according to Nelson (1930) and *cis*- and *trans*-aconitic acids by the methods of Bruce (1937) and Malachowski and Maslowski (1928). Esters were kindly supplied by Mr. G. K. Hughes, Chemistry Department, University of Sydney. Keto-acids were determined using the precautions adopted by Humphrey and Robertson (1953).

RESULTS.

Effect of substrates on respiration.

In addition to the compounds previously used (Humphrey, 1950), *cis*-aconitic and *iso*-citric acids were also tried. The former always inhibited (by as much as 50 p.c.) and the latter always increased O_2 consumption (by amounts reaching 70 p.c.). The inhibiting action of *cis*-aconitate was presumed due to the *in vitro* formation of a small amount of *trans*-aconitate before readings commenced. This was confirmed by the fact that 1 mM *trans*-aconitate could

[1]This continues the series which began in this Journal as an isolated paper (Humphrey, 1950).

[2]Working under a full-time grant from the Research Committee, University of Sydney.

cause inhibitions of up to 55 p.c. Such inhibitions are also obtained with other tissues (Saffran and Prado, 1949). With the other compounds, results similar to those previously given were obtained, except that malic acid sometimes (in six cases out of fifteen) produced an increase which ranged from 20 to 150 p.c.

The variation in activity shown in the presence of these compounds could be due to a variation in the metabolic activity of the sperm or to a variation in the permeability of the sperm to the compounds. To obtain more information on these points, several derivatives (mainly esters) of the compounds were used. These derivatives gave much more consistent results and indicated that the metabolic systems for these compounds were active in the sperm. For example, the following p.c. increases in O_2 consumption were found: ethyl citrate, 0–8; ethyl succinate, 115–325; ethyl malate, 50–60; ethyl acetate, 0; methyl acetate, 0; ethyl propionate, 0; methyl propionate, 0; ethyl butyrate, 20–55; methyl butyrate, 0–5; glucosamine, 15–30; hippuric acid, 12–30; glucose penta-acetate, 20–40; ethanol, 0, and methanol, 0. Many other compounds, e.g., ethyl oxalacetate, proved unsuitable in this type of work because of their rapid hydrolysis with consequent acidification of the suspensions.

Succinic dehydrogenase.

The sperm are very active in reducing methylene blue but this activity is not always increased in the presence of succinic acid. Although the first series of experiments (Humphrey, 1950) showed that succinate decreased the reduction time of methylene blue, subsequent work over five seasons has not been able to confirm this except in a few cases. As shown above, ethyl succinate is more consistent than succinic acid in increasing respiration, and this is also the case with regard to methylene blue reduction, i.e., ethyl succinate decreases the reduction time by amounts up to 40 p.c. Malonic acid, which is usually regarded as a specific inhibitor of succinic dehydrogenase, does not inhibit the endogenous O_2 consumption, but increases it by as much as 150 p.c. Pre-incubation of the suspensions with malonate (for periods up to one hour) did not alter the results. To check that the malonic acid is not acting without penetrating, methyl and ethyl malonate and ethyl ethylmalonate were also used. These esters acted consistently to give increased O_2 consumption, the increases ranging from 20 to 300 p.c. Malonate and its esters also increase the extra respiration brought about by methylene blue, e.g., in a typical experiment methylene blue increased respiration by 100 p.c., ethyl malonate by 120 p.c., and both together gave 260 p.c. Irrespective of the presence of methylene blue, succinic acid prevents the malonate increase, i.e., when added together, succinate and malonate keep respiration at the control level. In the presence of some other compounds (α-ketoglutarate, pyruvate, and, to a small extent, fumarate and malate), but without methylene blue, the malonate increase is further augmented, e.g., with the control respiration taken as 100, malonate gave intensities equal to 135, pyruvate 160, and both

together 190. With compounds which do not increase endogenous O_2 uptake (glucose, citrate, acetate, propionate and butyrate), there was no alteration in the increase due to malonate; with lactate, which increases O_2 consumption by about 20 p.c., the effect of malonate was to reduce this increase slightly.

Keto-acid metabolism.

The O_2 consumption of sperm suspensions is increased by the presence of keto-acids and experiments were carried out to see if the keto-acids were utilized or if, in the presence of other substrates, keto-acids accumulate in the suspension. When sperm are incubated with pyruvate, or α-ketoglutarate, no significant loss of these keto-acids occurs. In the presence of other compounds (lactate, citrate, *cis*-aconitate, acetate, propionate, butyrate, succinate and malonate), sometimes a faint trace, or usually no, keto-acid accumulated. There was some loss of citric acid on incubation, e.g., for a two-hour period in air the Q values were about 5.

DISCUSSION.

The respiratory effects obtained in the presence of organic acids show that the α-keto acids, oxalacetic and α-ketoglutaric are the most powerful in stimulating O_2 consumption. *Iso*-citric, pyruvic and sometimes malic acids were also able to increase O_2 consumption. Used as esters (which are thought to penetrate cells more readily than free acids, Beevers *et al.*, 1952), succinate, malate, and to a lesser extent butyrate and glucose, were also shown to stimulate O_2 consumption. These results contrast sharply with those of Spikes (1949), who found that the substrates usually associated with metabolism had no effect on the respiration of sea-urchin sperm, and further, that esters exerted an inhibiting action. In the experiments with esters, no mention was made of acidification due to possible hydrolysis of the esters.

Malonic acid (and its ester derivatives) increased the O_2 consumption of oyster sperm. This effect with malonic acid has previously been noted by Barron with sea-urchin sperm (Barron and Goldinger, 1941, found a 200 p.c. increase in O_2 uptake). On the other hand Spikes (1949) reported that malonic acid and ethyl malonate inhibited the respiration of sea-urchin sperm. Although malonate is usually regarded simply as an inhibitor of succinic dehydrogenase, there is evidence (Glass, 1951) that it can also dissociate oxidation from phosphorylation. If malonate does make oxidative phosphorylation in oyster sperm less efficient, then the increase in O_2 uptake becomes explicable. The addition of succinic acid (which has no effect by itself) to malonate-treated oyster sperm brought the respiration back to the control level, i.e., there was possibly some kind of a competitive effect between these two substances on the surface of the sperm which led to malonate being prevented from entering. Similar competition in other tissues has been shown by Cori (1926) for related pairs of substances, i.e., glucose and galactose; glycine and alanine. With sea-urchin

sperm, the addition of succinic acid (which alone increases respiration by 35 p.c.) to malonate-treated sperm left the respiration at the malonate level (Barron and Goldinger, 1941). Further, the malonate-treated oyster sperm which, as considered above, may have inefficient mechanisms for oxidative phosphorylation, is more affected by compounds which increase endogenous O_2 consumption (methylene blue, α-ketoglutaric acid, etc.) than control sperm, but is still unaffected by agents which do not give such increases (glucose, citrate, etc.).

The lack of disappearance of keto-acids when these are added to sperm suspensions suggests that these compounds act catalytically under those circumstances. Considering the results as a whole, it seems that the sperm can carry out many of the reactions of the citric acid cycle, provided that experimental conditions are adjusted to allow for differences in permeability.

SUMMARY.

The O_2 uptake of sperm suspensions was increased by most of the intermediates of the citric acid cycle. In some cases it was necessary to use esters of these compounds in order to obtain an activating effect, and it was postulated that the sperm is more permeable to these esters than to the corresponding free acids.

Malonic acid and its esters also stimulated O_2 uptake, probably due to a reduction in the efficiency of oxidative phosphorylation. Succinic acid prevented this stimulation, possibly because it stopped malonate entering. Substances which alone are able to stimulate O_2 uptake, further increased this malonate effect.

There is little, if any, accumulation of keto-acids in respiring sperm suspensions, nor is there significant breakdown of added keto-acids.

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THE METABOLISM OF OYSTER SPERMATOZOA

3. THE SEPARATION AND METABOLIC PROPERTIES OF SPERM HEADS AND TAILS

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Previous investigation of the metabolism of oyster spermatozoa (Humphrey, 1950; Humphrey and Jeffrey, 1954) has shown that these exhibit an active respiration but only slight glycolysis. The respiratory process involves a cytochrome system and is also dependent upon the presence of sulphydryl-containing compounds. Further, oxalacetic and α-ketoglutaric acids are able to increase the respiration greatly, whereas metabolically-associated acids (e.g., succinic) were without significant effect unless they were added as esters, i.e., in a form to which the sperm were permeable. It was suggested that the lack of effect of the non-esterified compounds might be due to the fact that they were not reaching the active parts of the sperm. To test this hypothesis, investigations were begun to see if it were possible to devise treatments which would allow sperm preparations to be made which would not suffer from this defect.

In this paper, results are presented of experiments carried out in order to examine the metabolism of isolated parts of the sperm.

METHODS.

Suspensions of whole sperm were made as before (Humphrey, 1950). The sperm consists of three main parts, i.e., head, neckbeads (midpiece) and tail, and although it was possible occasionally to remove some neckbeads, no method could be devised of obtaining suspensions of these neckbeads suitable for metabolic work. To obtain suspensions of heads (in reality heads plus neckbeads) or tails, the tails were first cut off by blending for 1 minute in a stainless steel micro-Waring blendor (Hoffstadt and Tripi, 1945) which had been modified by fitting an outer jacket in which alcohol at −10° C. was placed; this kept the temperature of the blendor contents between 0 and 5° C. The pH was then adjusted to 7·5 and the heads removed from the tails by centrifuging two or three times for 5 minutes at 2,500 r.p.m.

Homogenates were prepared by grinding between glass surfaces (Humphrey, 1946) for 1 to 2 minutes after the addition of a tenth of a volume of glass powder. The glass was then removed by centrifuging for 2 minutes at 500 r.p.m. To obtain whole sperm homogenates, either the suspension of sperm was directly homogenized or first broken into heads and tails. An outline of the procedures used is presented in Fig. 1; other methods used are those previously described (Humphrey, 1950).

[1]This investigation was supported by an apparatus grant from the Research Committee, University of Sydney.

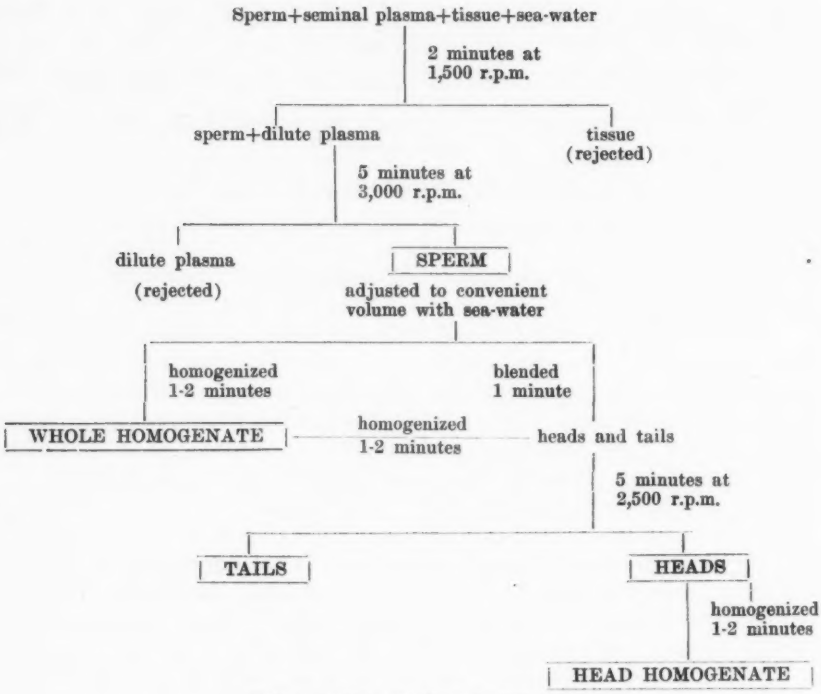

Fig. 1. Preparation of suspensions.

The composition of each fraction was always examined microscopically either with phase-contrast or after staining with Grübler's "Licht Grün." The blending removes all the tails, many of them being cut into several pieces. The heads become more clearly outlined (phase contrast) but are not changed in shape.

RESULTS.

Effect of phosphate. It was stated previously (Humphrey, 1950) that the concentration of phosphate buffer must be kept rather low to obtain the maximum respiration of intact sperm. This is not the case with the isolated heads and tails of the sperm (Table 1).

TABLE 1.

Effect of phosphate concentration on respiration of heads and tails.

Each flask contained either 1 ml. head suspension (=4·2 mg. N) or tail suspension (=1·6 mg. N) in sea water; Z_{O_2}=µl. O_2/mg. N/hr.

Phosphate (pH=7·3)	Z_{O_2} (heads)	Z_{O_2} (tails)
None	11·9	0·9
0·005 M	12·1	1·3
0·01 M	12·8	0·9
0·03 M	13·6	0·9

Intensity of endogenous respiration. As shown in Table 1, the intensity of respiration by the sperm heads is far greater than that shown by the tails. Results are now available for five breeding seasons and it is apparent that the respiratory intensity of the sperm alters as the season progresses. Usually, sperm suitable for metabolic work can be obtained towards the end of December and experiments can be continued until April or perhaps May. The onset of spawning is determined chiefly by rainfall and temperature changes. The 1950 season proved exceptionally long, and it was possible to obtain suitable sperm suspensions as late as July. Table 2 gives the results of respiratory measurements for that season on the various sperm preparations. The season has been arbitrarily divided into early (up to 31st March) and late (from 1st April).

TABLE 2.

Endogenous respiration of sperm fractions and effect of season.

The results are given as Z_{O_2} (μl. O_2/mg. N/hr.) in the presence of 0·008 M phosphate, pH=7·3. Standard errors are included.

Preparation	Early season	Late season
Sperm	19±1·9	27±4·3
Heads	10±0·7	23±3·7
Tails	2±0·8	3±1·4
Sperm homogenate	5±1·1	17±2·7
Head homogenate	5±0·9	12±3·2

Effect of substrates. The respiration of the various preparations was determined in the presence of several compounds at a concentration of 0·01 M; 8 mM phosphate buffer (pH=7·3) was also present. The compounds used were glucose, fructose, glycine, pyruvate, α-ketoglutarate, succinate, fumarate, malate, oxalacetate, formate, acetate, propionate, butyrate and lactate. Also, 0·01 mM cytochrome was tried.

The tail suspensions and the homogenates of whole sperm never gave an increased O_2 consumption in the presence of any of the above compounds. The head homogenates occasionally gave small (up to 15 p.c.) increases in the presence of pyruvate or succinate. The respiration of head suspensions was always increased by glucose (in 8 experiments), the increase amounting in one instance to 50 p.c. and in another to 100 p.c. Succinate gave only slight increases (maximum of 15 p.c.). Ketoglutarate regularly gave up to 50 p.c. increase; malate and oxalacetate sometimes gave up to 50 p.c. increase.

Succinic dehydrogenase. Methylene blue (0·1 mM) was quickly reduced by head suspensions and also increased O_2 uptake by 10-75 p.c. The rate of reduction of methylene blue was unchanged by the addition of succinate. However, 0·01 M ethyl succinate increased the speed of reduction by about 25 p.c. Ethyl succinate consistently increased O_2 uptake by 50 to 60 p.c. Malonate, ethyl malonate and ethyl ethylmalonate gave increases of 10 to 20 p.c. in O_2 uptake.

DISCUSSION.

The seasonal influence on the respiratory activity of the sperm is probably an indication of the sexual maturity of the animals used. Carter (1931) working with sea-urchin sperm, has previously noted that the maturity of the animals influenced the type of O_2 consumption but not the value of the Z_{O_2}/mg. N, i.e., sperm from "perfectly mature" animals showed an O_2 uptake linear with time, but if animals not quite mature were used, then a preliminary period of 20 to 40 minutes in the experimental fluid or of 3 hours in the "dry" (undiluted) state was necessary before linear O_2 uptakes were obtained. No such effect was obtained in the present series of experiments, the seasonal variation being simply one in respiratory intensity, possibly due to the fact that sperm taken at the height of the season (autumn) are more complete in their metabolic equipment.

Zittle and Zitin (1942) have shown that in bull sperm, fragmented by ultrasonic vibrations or by grinding with glass powder, the respiratory activity resides principally in the tail, the head having almost no activity. The reverse is true for oyster sperm (Table 2) and the processes used for separation bring about a 5-35 p.c. loss in respiratory ability, depending on the ripeness of the animals used. The treatment also lowers the ability of the sperm to metabolise added substrates, i.e., keto-acids are less active in increasing O_2 uptake with head suspensions than they are with whole sperm, and there is no increase in the range of compounds metabolized. Breaking the isolated heads by homogenization almost completely destroys any ability to use added compounds and also removes 50 p.c. of the O_2 uptake. Although cytochrome C was ineffective in restoring the respiratory ability of head suspensions, methylene blue had a moderate action, much smaller however than had previously been shown with whole sperm. Succinic dehydrogenase was shown to be present in head suspensions by using ethyl succinate as a substrate. The increases in O_2 uptake found previously (Humphrey and Jeffrey, 1954) with whole sperm treated with malonic acid or its esters were obtained to a smaller degree with head suspensions.

Thus it was not found possible to use mechanical disintegration to obtain suspensions of parts of the sperm which reacted actively to added substrates; the metabolic effects obtained were those previously found but were now smaller in intensity.

SUMMARY.

Oyster sperm were separated into heads and tails by means of the Waring blendor followed by differential centrifugation. Depending on the sexual maturity of the animals, the head fraction showed 50-90 p.c. of the respiratory activity of the whole sperm whereas the tails showed only 10 p.c. If the whole sperm were homogenized between ground glass surfaces, 20-60 p.c. of the activity was retained; if head suspensions were similarly homogenized, only 20-40 p.c. remained.

The substrates and related compounds previously used on whole sperm had quantitatively less effect on these preparations, but the qualitative picture was similar.

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THE METABOLISM OF OYSTER SPERMATOZOA

4. THE EFFECT OF LOW pH ON OYSTER SPERMATOZOA

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Investigation of the metabolism of oyster spermatozoa posed the problem of the permeability of the sperm to metabolically important compounds (Humphrey, 1950) and subsequent work on the constituent parts of the sperm (Humphrey and Pollak, 1954) and on the effect of esterified substrates (Humphrey and Jeffrey, 1954) left some problems unanswered. This was so because the respiratory activity resides largely in the head, and the methods which were developed for disintegrating the head reduced the respiration greatly and failed to give consistent increased O_2 consumption in the presence of added possible substrates.

The next approach was to try other methods which might either separate parts of the sperm (and allow the repetition of the previous experiments to see if this new material was more active) or which might render the sperm more permeable. Various treatments were used, the most successful of which was the addition of dilute hydrochloric acid.

Because it is known (Tyler and O'Melveny, 1941) that dilute acid extracts gamones from the sperm of other species, the acid extracts of oyster sperm were tested to see if any substance was removed from the sperm or if the effect of HCl was simply one by which the physical structure of the sperm was changed.

METHODS.

Separation into constituent parts.

Exposure to different solutions. The sperm suspension was centrifuged for 5 minutes at 3,000 r.p.m. and the "solid" sperm resuspended in the appropriate solution for either 30 or 60 minutes. After this, the sperm were centrifuged as before and resuspended in sea-water.

Freezing and thawing. A centrifuge tube containing 5 to 6 ml. sperm suspension was immersed in an alcohol bath at −15° C. for varying periods and then thawed in a bath at either 25° C. or 37° C.

Ultrasonic vibrations. Sperm suspensions were exposed to vibrations (from a magnetostriction oscillator) of sufficient intensity to produce vigorous cavitation. Periods of up to 8 minutes were used; no rise in temperature was observed.

[1]This investigation was supported by an apparatus grant from the Research Committee, University of Sydney.

Attempts to increase permeability.

Exposure to different solutions. The sperm suspension was centrifuged for 5 minutes at 3,000 r.p.m. and the "solid" sperm resuspended in the appropriate solution for either 30 or 60 minutes. After this, the sperm were centrifuged as before and resuspended in sea-water.

Cold treatment. Sperm suspensions were kept at 4 to 5° C. for 30 minutes or 24 hours.

Acid treatment. Sperm suspensions were acidified to various pH values as determined by the glass electrode. After 1 hour the sperm were centrifuged off and resuspended in sea-water, the mixture finally being adjusted to pH 7·3.

Ultra-violet absorption data were obtained on a Beckman, DU Spectrophotometer. Phosphorus was estimated according to Allen (1940), nitrogen by direct Nesslerization of H_2SO_4-H_2O_2 digests and ribose and desoxyribose by the orcinol and diphenylamine methods given by Morse and Carter (1949).

Other methods were those previously given (Humphrey and Pollak, 1954). At all stages of handling, the sperm were examined microscopically using high-power and oil-immersion phase-contrast or after staining with "light green" or "neutral red."

RESULTS.

Separation of parts.

Table 1 gives an outline of the various methods tried in order to separate the sperm head from the tail. In addition, the effect of the treatments on the morphology, motility and endogenous respiration is given.

TABLE 1.

Effect of various treatments on morphology, motility and respiration.

Treatment	*Morphology and motility*	*Respiration*
Distilled water for 30 or 60 minutes	Tails curl up but elongate again in sea-water. In some experiments only heads were visible, but no satisfactory separation into heads and tails on centrifugation.	Usually zero or very low.
0·01 M KH_2PO_4-Na_2HPO_4 (pH 7·3) for 30 or 60 minutes	Do not stain so heavily, tails not well defined. Otherwise as with water.	Usually zero or very low.
0·01 M citric acid in 0·25 M NaCl, or 0·25 M citric acid for 30 minutes	Coagulum formed and motility lowered, morphology unchanged.	Zero.
Frozen for 30 minutes	Morphology unchanged, motility increased.	Increased 20-100 p.c.
Frozen for 1, 2 or 24 hours	Sperm agglutinated, nearly all dead.	Zero.
Ultra-sonic vibration	Motility slightly increased, morphology unchanged.	Unaffected.
0·001 M $HgCl_2$ for 30 minutes	Tails curl up, but elongate again in sea-water. Do not stain so darkly with "light green."	No O_2 uptake.
0·01 M $CaCl_2$ for 30 minutes	Sperm settle when resuspended in sea-water.	Lowered O_2 uptake. No substrate effect.

TABLE 1 (continued).

Treatment	*Morphology and motility*	*Respiration*
Mixture of 0·25 M NaCl and KCl for 30 minutes:		
NaCl:KCl=1:0	Immotile; morphology unchanged.	No O_2 uptake.
NaCl:KCl=9:1	Motility diminished, morphology unchanged.	No change.
NaCl:KCl=0:1	Feeble motility; morphology unchanged.	No O_2 uptake.
Mixtures of isotonic NaCl and KCl for 30 minutes:		
NaCl:KCl=49:1	Feeble motility; morphology unchanged.	O_2 uptake almost zero.
NaCl:KCl=29:1	Motility and morphology unchanged.	Lowered O_2 uptake; no substrate effect.
NaCl:KCl=9:1	Motility and morphology unchanged.	No change.
NaCl:KCl=1:1	Motility and morphology unchanged.	No change.
NaCl:KCl=1:9	Motility and morphology unchanged.	Lowered O_2 uptake; no substrate effect.
Cold treatment: 5° C. for 30 minutes	Motility and morphology unchanged.	Increased O_2 uptake; no substrate effect.
4° C. for 24 hours	Motility and morphology unchanged.	Lowered O_2 uptake; no substrate effect.
Acid treatment: 0·1 N HCl added to give stated pH then mixture left 60 minutes.		
pH=3·5	Coagulum formed which could not be resuspended.	No O_2 uptake.
pH=4·5	Motility lost and jelly formed. On resuspension some motility regained and most of jelly dispersed.	Lowered O_2 uptake, increased by fructose and succinate.
pH=4·8	Usually no motility and the sperm clot; on resuspension clot dispersed and nearly all motility regained.	Lowered O_2 uptake, increased by fructose and succinate.
pH=5·5 and 6·0	No clotting, motility slightly reduced; on resuspension motility restored.	Slightly lowered O_2 uptake; only occasional increases with fructose and succinate.

Thus, no new method was found for separating the heads and tails.

Alterations to permeability.

The next step was to devise treatments which would change the sperm so that possible substrates could enter, be metabolized and increase the O_2 consumption. For these experiments succinic acid and fructose were chosen as test substances. Table 1 shows the result obtained with these procedures.

It can be seen that of all the treatments tried only one was able to give motile suspensions with good O_2 uptake which was increased in the presence of fructose or succinate. The effect was not produced specifically by HCl because acetic acid gave similar effects. It is important not to over-acidify the suspensions because then a clot is formed which has to be discarded on resuspension. Also the activity of the sperm which are left is much reduced, both with regard to their O_2 uptake and any increase brought about by the addition of substrates. In practice then, the procedure is best carried out by taking 5 to 10 ml. of suspension and adding 0·1 N HCl drop by drop until the pH is between 4·6 and 4·9; this point can be judged macroscopically by observing incipient clotting. The pH is then measured with a glass electrode and the mixture allowed to stand for 1 hour; during this period the pH rises for the first few minutes to about pH 5·0 when it remains steady. Resuspension is best carried out by the gradual addition of sea-water to the centrifuged "acid" sperm until the desired volume is reached; the pH is then about 6·5-7·0. Finally, 0·1 N NaOH is carefully added with immediate mixing until pH 7·3 is reached; if excess NaOH is added a tenacious coagulum is obtained. The respiratory intensity of acid-treated sperm is 40-85 p.c. of untreated sperm.

The substrate effects obtained as a result of the acid treatment are interpreted as demonstrating an increase in permeability because many of the compounds which now increase O_2 uptake (see below) have that action on untreated sperm only if the compounds are added as esters, a form to which cells are readily permeable (Humphrey and Jeffrey, 1954).

Effect of substrates on acid-treated sperm.

Several compounds were used in an attempt to see which could influence the respiration of the acid-treated sperm. The increases observed varied from 10-100 p.c. Only one compound (*cis*-aconitate) failed to give an increase at any time and this is possibly due to the formation of some *trans*-aconitate which inhibits sperm respiration (Humphrey and Jeffrey, 1954). Fructose, butyrate and glycine invariably gave increases in O_2 uptake, while glucose, succinate, pyruvate, fumarate and malate usually did so. Occasionally, increases were observed with acetate, propionate and citrate; *iso*-citrate, lactate, oxalacetate and α-ketoglutarate gave only small increases.

Succinic dehydrogenase.

Methylene blue is reduced by suspensions of acid-treated sperm at about half the rate obtained with whole sperm. The rate of reduction is not increased by succinate or ethyl succinate. However, ethyl succinate produces a 25 p.c. increase in O_2 uptake. Methylene blue is very active in increasing O_2 uptake, usually by 100 p.c. (from 50 to 300 p.c.). Where high activation occurs there is

a steady falling off over the experimental period (1 to 2 hours). Malonate produces no effect on O_2 uptake, except occasionally about a 10 p.c. decrease; ethyl malonate and ethyl ethylmalonate increase O_2 uptake by 30 to 200 p.c., the effect decreasing with time.

TABLE 2.

Composition of seminal plasma and acid supernatants.

The figures are given as mg./100 ml. original fluid. Those in brackets are calculated from ultra-violet absorption data, assuming the compounds are nucleic acids.

	Seminal plasma		Acid supernatant	
No.	1	2	1	2
Ribose				
First extract	—	—	—	—
Final extract	0·12	0·15	0·15	0·12
Desoxyribose				
First extract	3·00	1·42	3·00	1·99
Final extract	0·10	0·10	3·30	3·68
Inorganic phosphate				
First extract	2·02	1·92	2·02	1·53
Final extract	—	0·03	—	0·06
Organic phosphate				
First extract	0·32	0·32	0·76	0·50
Final extract	0·08+(0·05)	0·06 (0·12)	0·63+(0·58)	0·60 (0·64)

+ uncorrected for traces of inorganic phosphate.

Supernatants from acid-treated sperm.

When the sperm were centrifuged from the acidified sea-water, a slightly cloudy fluid was left. Residual sperm were removed by centrifuging at 0° C. for 10 minutes at 8,000 *g*. The ultra-violet absorption of these solutions brought to pH 7·7 usually showed a peak (occasionally a plateau) at 260–268 mμ., with a subsidiary broad peak at 320–340 mμ. "Seminal plasma," i.e., the fluid obtained after centrifuging the initial sea-water extract of the gonads, gave similar absorption curves but on a much reduced scale (less than one-third). These results suggested that a nucleoprotein (or derivative) was extracted by the HCl. To test this further, solutions were analysed according to the Schneider (1945) fractionation procedure (Table 2); no values are given for ribose in the first extract since these develop a brown colour after heating. The results indicate that the initial trichloracetic acid extract from both the seminal plasma and the acid supernatants contained ribose and desoxyribose compounds, inorganic phosphate and organic phosphates, the acid supernatants giving more organic phosphate than the seminal plasma. Both extracts showed absorption maxima at 279 and 330 mμ. The seminal plasma in the final trichloracetic acid extract gave only traces of sugars and phosphates whereas the acid supernatants yielded, in addition to traces of ribose and inorganic phosphate, significant quantities of desoxyribose and organic phosphates. Neither

sample in this final trichloracetic acid extract showed absorption at 330 mμ, the seminal plasma showed a faint peak at 268-275 mμ, and the acid supernatant showed a marked peak at 268 mμ. On the assumption that all the organic phosphorus in this final trichloracetic acid fraction comes from nucleic acid, a comparison has been made in the Table between the organic phosphorus found and that expected from the ultra-violet absorption; the comparison shows very good agreement in the case of the acid supernatants. These results show that a desoxynucleic acid or derivative has been extracted from the sperm during the treatment with acid sea-water.

Further, it was shown that this desoxyribose compound could be precipitated by dialysis. For example, when 5 ml. of acid supernatant were dialysed for 24 hours against 0·1 N, pH 4·6 acetate buffer, a white precipitate was obtained. Analysis by the procedure given above showed that the precipitate contained all the desoxyribose of the original material (8·0 mg. p.c.) and all the organic phosphate (1·0 mg. p.c.); further, the final trichloracetic acid extract of the precipitate gave a sharp absorption maximum at 265 mμ, accounting for all of such absorption present originally.

DISCUSSION.

Most of the methods used here to separate the constituent parts of the sperm have been used by other workers, with varying degrees of success, on the sperm of other animals, e.g., Miescher (1896) used distilled water with fish sperm, Pollister and Mirsky (1946) and Hultin (1947) used citric acid solutions on sea-urchin sperm, and Henle *et al.* (1938) used ultrasonic vibrations on mammalian sperm. All of these methods were ineffective with oyster sperm, and, in addition, many of them destroyed all respiratory ability. The next procedure was to attempt to make the sperm more permeable by removing layers from it or rendering any boundary membranes less stable. Following the observation of Chambers (1940) that Ca is necessary for the stabilization of the hyaline layer of sea-urchin eggs, the effect of various concentrations of salts was tried. Further, Chambers and Kempton (1933) have shown that exposure to moderately low temperatures increased the permeability of chicken mesonephra. Both of these types of treatment proved ineffective with oyster sperm. Finally a modification of the method of Tyler and O'Melveny (1941) for removing anti-fertilizin from sea-urchin sperm was used. This method originally consisted of acidifying the suspension to a pH between 3·5 and 6·0, allowing to stand for 1 hour, bringing to pH 7·9 and then centrifuging. These workers found that the fertilizing capacity of the sperm was considerably reduced with, usually, only a moderate reduction in respiratory ability.

With oyster sperm, it was found that during the acidification period a desoxyribonucleoprotein or derivative is extracted. This component resembles

that obtained by Runnström *et al.* (1942, 1944) during their investigations on gamones in sea-urchin sperm. By qualitative tests on hot sea-water extracts of frozen sperm, they showed the presence of desoxyribose and organic phosphate; the extracts showed an absorption band near 260 mμ, and contained a component whose molecular weight was in the range of $2\text{-}10\times10^3$. Further, Tyler (1949) has shown that in sea-urchin sperm, the anti-fertilizin is located laterally around the head, in the region between the acrosome and the midpiece. Therefore it seems that in this region a desoxyribose derivative occurs and that this compound is removed by dilute acid or hot sea-water. Associated with the removal of this compound by acid treatment of oyster sperm is an alteration in the respiratory behaviour. It is possible, but not certain, that this alteration is directly related to the removal of this component and not due simply to a physical effect.

From the observations made of the effect of substrates on the respiration of the acid-treated sperm, it seems that the systems most active in untreated sperm, e.g., those oxidizing α-ketoglutarate, oxalacetate, etc., no longer operate, while those for other substrates are still functional, and can be demonstrated. Also the rate of methylene blue reduction is reduced to about half the value found with control sperm. Combining the results from all the types of treatment used, it can be concluded that the sperm are able to metabolize an extensive range of compounds. However, the importance of these metabolic pathways under natural conditions is still obscure. In the case of mammalian sperm, the substrates which supply energy for motility are fructose (contributed by accessory gland secretions) and phospholipide (laid down in the sperm during its formation). In oyster sperm there is no fructose, but phospholipide is present and disappears during incubation (Humphrey, 1950). The metabolic systems brought into evidence since then could be interpreted as pathways by which the phospholipide molecule is used.

SUMMARY.

Several different treatments were investigated in an endeavour to increase the permeability of oyster sperm to substrates; most of them were unsuccessful.

However, allowing sperm suspensions to stand at low pH decreased the endogenous respiration but allowed substrate effects to be demonstrated more clearly than with untreated sperm.

This acid treatment removed a desoxyribose derivative from the sperm.

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